CRANFIELD UNIVERSITY

ILONA G. PILLAI

IDENTIFICATION AND CARCINOGENICITY OF POLYCYCLIC AROMATIC HYDROCARBONS IN TRANSFORMER OIL

CRANFIELD CENTRE FOR ANALYTICAL SCIENCE
INSTITUTE OF BIOSCIENCE AND TECHNOLOGY

PhD THESIS

MAY 2003

ABSTRACT

Insulating oils are rich in polycyclic aromatic hydrocarbons (PAHs) which act as inhibitors of oil breakdown, but are believed to be the main source of oil mutagenicity when converted to their epoxide form by mammalian enzymes. The current industry-recognised measurement of oil risk, the IP 346 method, measures total aromatic content and therefore cannot be directly related to PAH risk. This thesis describes efforts made to establish the contribution of PAH species alone to total oil mutagenicity.

The aromatic fraction of the oil was successfully extracted with the Grimmer and Blackburn methods. When tested with 80% v/v S-9 (activation enzymes), the Ames test results of these extracts correlated with IP346 data in that oil 4 (low IP346 value) gave no indirect mutagenicity, whilst Nytro-10GBN (intermediate IP346 value) gave a 40% increase in revertants and oil 8 a 40-50% increase (high IP346 value). In the absence of S-9 however, both methods produced ~800-1000 revertants for all oil samples. This indicated an additional threat from direct mutagens in these oils. However the source of mutagenicity remained inconclusive due to extract complexity. A new extraction that was specific to PAHs was therefore developed using solid phase extraction as a rapid, safer, more repeatable system with potential for automation.

The efficiency of the new C18/Silica/Isolute PAH HC extraction method was 68-84% for the carcinogenic EPA priority PAHs and 21-67% for non-mutagenic priority PAHs, with the most volatile PAHs being lost during the evaporation step. The GC-MS of these extracts was successfully used to identify and quantify these compounds in the oil by total peak area integration, offering an alternative to the IP 346 method. Ames test studies showed indirect (essentially PAH) mutagenicity (~70-100 revertants) only for the C18/Silica/Isolute PAH HC extracts indicating that the extracts were PAH specific. Artificial ageing of the oil was found to alter PAH composition, although no change in oil mutagenicity was observed.

ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisors Dr Steve Setford (CCAS) for all his help and support and Professor Selly Saini (CCAS) for his encouragement. Many thanks also go to Dr Dave Aldred for his help in setting up the bacterial tests. Thanks go to Dr Richard Heyward, Dr Gordon Wilson and Professor Bruce Pahlavanpour from the National Grid Company Plc for their expertise, and the financial assistance that supported this work. In addition, financial support from a Total Technology EPSRC studentship is gratefully acknowledged.

Many thanks also go to the wonderful friends I have made during my years at Cranfield, in particular to Drs Mike Malacha, Laurie Ritchie and Andrew Moody who kept me smiling in the lab. Much love also goes to Jude Taylor, Kal Karim, Kate Hipwood and Jane Rixon, my lunch, curry and running partners. Particular heartfelt thanks go to Dr Damian Baskeyfield to whom I aspire, for always bringing out the best in me. Finally, I would like to acknowledge the unending love and support shown to me by my family for which I am truly grateful. I dedicate this work to you; Mum, Dad and Meena.

CONTENTS

ABSTRACT ..... **I**

ACKNOWLEDGEMENTS ..... **II**

LIST OF FIGURES ..... **XI**

LIST OF TABLES ..... **XV**

LIST OF PLATES ..... **XVIII**

LIST OF APPENDICES ..... **XVIII**

NOTATION ..... **XIX**

CHAPTER 1.0 INTRODUCTION AND LITERATURE REVIEW **1**

1.1 TRANSFORMERS AND TRANSFORMER OIL **2**

1.1.1 COMPOSITION OF TRANSFORMER OIL 2

1.1.2 THE BREAKDOWN OF OIL 3

1.1.3 CARCINOGENICITY OF THE OIL 4

1.1.3.1 PAH Mutagenicity Versus Aromatic Mutagenicity *4*

1.2 POLYCYCLIC AROMATIC HYDROCARBONS **5**

1.2.1 PAH STRUCTURE 6

1.2.2 CARCINOGENIC AND MUTAGENIC PAHS 7

1.2.3 PAH ACTIVATION 7

1.2.4 IARC CLASSIFICATION OF PAHS 10

1.3 TESTS FOR CARCINOGENICITY **12**

1.3.1 MUTAGENICITY AS A MEASURE OF CARCINOGENICITY 13

1.3.2 PAH TOXICITY 14

1.4 AIMS OF THESIS **15**

1.4.1. CONSTRAINTS OF WORK 16

1.4.1.1 The End-User's Requirements *17*

1.4.1.2 Oil Extracts for Both PAH Mutagenicity and Analysis *17*

1.4.1.3 Regulatory Concerns *18*

1.4.1.4 Variation in PAH Content with Oil Origin and Age.....18
1.4.1.5 PAH Mixtures in a Complex Matrix.....18
1.4.1.6 Volume of Oil for Testing.....19
1.5 INTRODUCTION TO METHODOLOGIES.....**20**
1.5.1 THE AMES TEST FOR MUTAGENICITY.....20
1.5.1.1 Bioactivation of Chemical Carcinogens in the Ames Test.....22
1.5.1.2 Key Factors and Modifications of the Ames Test.....23
1.5.1.3 Alternative Mutagenicity Tests.....24
1.5.2 LIQUID-LIQUID EXTRACTION.....24
1.5.3 SOLID PHASE EXTRACTION.....25
1.5.3.1 Types of SPE.....26
1.5.3.2 Effects of Length and Width on SPE Columns.....28
1.5.3.3 Sample Breakthrough.....29
1.5.4 GAS CHROMATOGRAHY MASS SPECTROSCOPY (GC-MS).....29
1.5.4.1 Gas Chromatogram (GC).....30
1.5.4.2 Mass Spectrometer (MS).....34
1.5.5 IMMUNOASSAY.....35
CHAPTER 2.0 MATERIALS AND METHODS.....**37**
2.1 MATERIALS.....**38**
2.1.1 OILS SAMPLES.....38
2.1.2 THE AMES TEST.....39
2.1.2.1 Inoculation of Bacteria from Original Freeze Dried Sample.....40
2.1.2.2 Preparation of Minimal Glucose Vogel Bonner (VB) Plates.....40
2.1.2.3 Preparation of the S-9 Mix.....41
2.1.2.4 Preparation of Top Agar.....42
2.1.3 LIQUID-LIQUID EXTRACTIONS.....42
2.1.4 SOLID PHASE EXTRACTION.....42
2.1.5 TOXICITY MEASUREMENTS.....43
2.1.6 IMMUNOASSAY.....44
2.2 METHODS.....**44**
2.2.1 AMES TEST SAMPLE PREPARATION.....44

2.2.2 THE AMES TEST PROCEDURE 46
2.2.3 LIQUID-LIQUID EXTRACTIONS 47
2.2.3.1 IP 346 47
2.2.3.2 The Grimmer Method 47
2.2.3.3 The Blackburn Extraction 48
2.2.4 SOLID PHASE EXTRACTION 48
2.2.4.1 SPE with 15 g Columns 48
2.2.4.2 SPE with 182 g Columns 49
2.2.4.3 SPE with 1 g Columns 50
2.2.5 INVESTIGATION OF OIL INTERFERENCE WITH LIQUID MEDIA AMES TEST 50
2.2.6 GC-MS ANALYSIS 51
2.2.6.1 Perkin Elmer System 51
2.2.6.2 Hewlett Packard System 52
2.2.7 THE ISOLUTE PAH HC PROTOCOL 53
2.2.7.1 Manufacturer's Method 53
2.2.7.2 Modified Method 54
2.2.8 THE MINERAL INSULATING OIL FINGERPRINTING TECHNIQUE 54
2.2.9 C18/SILICA/ISOLUTE PAH HC EXTRACTION 54
2.2.10 LARGE SCALE C18/SILICA/ISOLUTE PAH HC 56
2.2.11 TOXICITY TESTING OF OILS AND OIL EXTRACTS WITH *S. TYPHIMURIUM* 57
2.2.12 THE MINISCREEN MUTAGENICITY ASSAY 57
2.2.13 IMMUNOASSAY RAPID ASSAY PAH TEST KIT 58

CHAPTER 3.0 RESULTS: MEASURING MUTAGENICITY OF POLYCYLIC AROMATIC HYDROCARBONS IN OILS AND OIL EXTRACTS 60

3.1 INTRODUCTION 61

3.2 AMES TESTING OF EPA 16 PRIORITY PAH STANDARD 62
3.2.1 EFFECTS OF DMSO ON *S. TYPHIMURIUM* 62
3.2.1.1 Mutagenicity of Individual EPA 16 Priority PAHs 63
3.2.1.2 Mutagenicity of EPA 16 Priority PAH Mixture 66

3.3 AMES TESTING OF WHOLE OIL 67
3.3.1 DIRECT ADDITION OF OIL 67
3.3.2 OIL WITH TWEEN 80 68

3.3.3 INVESTIGATION OF WHOLE OIL INTERFERENCE ON THE AMES TEST 69

3.3.3.1 The Impact of Oil on S-9 Activity *72*

3.3.3.2 The Impact of Oil on S-9 Activity and S. Typhimurium *with Liquid Ames Test* *72*

3.4 AMES TESTING OF OIL EXTRACTED BY LIQUID-LIQUID SEPARATION 74

3.4.1 IP 346 EXTRACTION AND GRIMMER EXTRACTION 75

3.4.1.1 Total Ion Chromatogram (TIC) of Grimmer and IP 346 Extracts 80

3.4.2 THE GRIMMER METHOD AT 80% S-9 81

3.5 AMES TESTING OF AGED OIL GRIMMER EXTRACTS 82

3.6 AMES TESTING OF FRACTIONATED SOLID PHASE EXTRACTS 84

3.6.1 OPTIMISATION OF OIL VOLUME FOR SILICA COLUMN EXTRACTION 85

3.6.2 MUTAGENICITY OF SOLID PHASE EXTRACTS 86

3.7 AMES TESTING OF GRIMMER/SPE EXTRACTS 90

3.8 INVESTIGATION OF OIL EXTRACT INTERFERENCE ON THE AMES TEST 92

3.9 CONCLUSIONS 96

CHAPTER 4.0 RESULTS: DEVELOPMENT OF A POLYCYCLIC AROMATIC HYDROCARBON EXTRACTION METHOD FOR TRANSFORMER OILS USING GC-MS ANALYSIS 97

4.1 INTRODUCTION 98

4.1.2 SOLID PHASE EXTRACTION METHODS FOR PAH ANALYSIS 98

4.1.2.1 Alternative Solvents 99

4.1.2.2 Column Size and Oil Volume 99

4.2 DEVELOPMENT OF SILICA SOLID PHASE EXTRACTION 100

4.2.1 AMOUNT OF OIL USED FOR EXTRACTION 101

4.2.2 MEASURING NATURALLY OCCURRING PAHS IN WHITE "BLANK" OIL WITH SILICA EXTRACTION 101

4.2.2.1 Solvent Selection for Solid Phase Extraction 103

4.2.2.2 Silica Sorbent Activation 104

4.2.2.3 Variation on Column Dimension 106

4.2.2.4 Flow Rate *108*

4.3 ALTERNATIVE SORBENTS **108**

4.3.1 ALUMINIUM OXIDE (ALUMINA) 108

4.3.2 USE OF CYANOPROPYL SORBENT AS AN EXTRA SAMPLE CLEAN-UP STEP 110

4.3.3 ISOLUTE PAH HC COLUMN 112

4.4 MULTI-STEP SPE FOR PAHS IDENTIFICATION IN OILS **115**

4.4.1 OIL FINGERPRINTING 116

4.5 DEVELOPMENT OF THE SPE C18/SILICA/ISOLUTE PAH HC METHOD FOR PAHS **118**

4.5.1 LIQUID-LIQUID EXTRACTION FOLLOWED BY ISOLUTE PAH HC COLUMN 119

4.5.2 C18 WITH ISOLUTE PAH HC 122

4.5.3 COMBINED C18-SILICA SPE FOR OIL CLEAN-UP 123

4.5.4 C18/SILICA/ISOLUTE PAH HC 124

4.6 TESTING THE C18/SILICA/ISOLUTE PAH HC EXTRACTION FOR EFFICIENCY AND REPEATABILITY **125**

4.6.1 LOSS OF PAHS FROM EVAPORATION 130

4.6.2 TOTAL LOSS OF PAHS FROM THE C18/SILICA/ISOLUTE PAH HC 131

4.6.3 EXTRACTION EFFICIENCY OVER A RANGE OF SPIKE LEVELS 133

4.7 CONCLUSIONS **134**

CHAPTER 5.0 RESULTS: THE BLACKBURN AND C18/SILICA/ISOLUTE EXTRACTION METHODS WITH MUTAGENICITY AND TOXICITY TESTS **135**

5.1 INTRODUCTION **136**

5.2 AMES TESTING OF BLACKBURN EXTRACTS WITH 80% **137**

S-9 ..... **137**

5.3 MINISCREEN TEST FOR C18/SILICA/ISOLUTE PAH HC EXTRACTS **139**

5.4 CHANGES IN PAH CHROMATOGRAMS FOR LARGE SCALE C18/SILICA/ISOLUTE EXTRACTION **142**

5.5 AMES TESTING OF C18/SILICA/ISOLUTE PAH HC EXTRACTS **145**

5.5.1 TESTING COLUMN RESIDUE FOR INTERFERENCE 145

5.5.2 MUTAGENICITY OF OIL WITH LARGE SCALE C18/SILICA/ISOLUTE PAH HC EXTRACTION WITH 80% S-9 146
5.5.3 MUTAGENICITY OF NYTRO-10GBN WITH SMALL SCALE C18/SILICA/ISOLUTE PAH HC EXTRACTION WITH 80% S-9 149
5.6 AMES TESTING OF BLACKBURN AND C18/SILICA/ISOLUTE PAH HC EXTRACTS OF AGED OILS 150
5.6.1 BLACKBURN EXTRACTS OF AGED OILS WITH 80% S-9 150
5.6.2 C18/SILICA/ISOLUTE PAH HC EXTRACTS OF AGED OIL WITH 80% S-9 152
5.7 INVESTIGATION OF OIL EXTRACT INTERFERENCE ON THE AMES TEST 154
5.7.1 INHIBITION AT 80% S-9 157
5.8 TOXICITY OF OIL EXTRACTS 158
5.8.1 TOXICITY OF OIL 8, 4, NYTRO-10GBN AND WHITE OIL EXTRACTS 160
5.8.2 TOXICITY OF AGED OIL EXTRACTS 162
5.9 CONCLUSIONS 165
CHAPTER 6.0 RESULTS: MEASURING VARIATION IN POLYCYCLIC AROMATIC HYDROCARBON CONTENT WITH THE C18 SILICA ISOLUTE EXTRACTION IN OILS 166
6.1 INTRODUCTION 167
6.1.1 COMPARISON OF QUALITATIVE MEASUREMENT 167
6.1.2 COMPARISON OF TOTAL AROMATIC MEASUREMENT 168
6.2 IMMUNOASSAYS AS A QUANITATIVE DETECTION METHOD 169
6.2.1 EFFECT OF ENVIRONMENT ON IMMUNOASSAY 170
6.2.2 TESTING THE IMMUNOASSAY KITS FOR ACCURACY 171
6.2.2.1 Total Immunoassay Kit 172
6.2.2.2 Carcinogenic Immunoassay Kit 173
6.2.3 TOTAL AND CARCINOGENIC PAH CONTENT OF OILS WITH IMMUNOASSAY ...174
6.3 GENERAL TRENDS IN PAH CONTENT OF TRANSFORMER OILS ...177
6.3.1 VALIDATION OF THE C18 SILICA ISOLUTE PAH HC EXTRACTION METHOD FOR TOTAL PAH DETERMINATION 179
6.4 IDENTIFICATION OF PAHS IN TRANSFORMER OIL 182

6.4.1 INSULATING MINERAL OIL FINGERPRINTING COMPARED TO C18/SI/ISOLUTE EXTRACTS 182
6.4.2 IDENTIFICATION OF PAHS IN OIL 1 TO 12 184

6.5 QUANTITATION OF EPA 16 PRIORITY PAHS IN EACH OIL 186

6.6 EFFECTS OF AGEING ON PAHS IN OIL 191
6.6.1 QUALITATIVE VARIATIONS OF PAH IN AGED OILS 191
6.6.2 QUANTITATIVE VARIATIONS OF PAH IN AGED OILS 193

6.7 USE OF C18 SILICA ISOLUTE PAH HC EXTRACTION WITH NON-TRANSFORMER OILS 195

6.8 CONCLUSIONS 199

CHAPTER 7.0 DISCUSSION AND FURTHER WORK 200

7.1 INTRODUCTION 201
7.1.1 AIMS AND APPROACHES OF THE WORK 202

7.2 MUTAGENICITY OF PAHS IN OIL 205
7.2.1 AMES TESTING OF EPA 16 PAH STANDARD 205
7.2.2 AMES TESTING OF WHOLE OIL 206
7.2.3 AMES TESTING OF IP 346 AND GRIMMER EXTRACTS AT 10% S-9 207
7.2.3.1 Grimmer Method 209
7.2.3.2 IP 346 Method 210
7.2.4 AMES TESTING OF GRIMMER EXTRACTS AT 80% S-9 211
7.2.5 AMES TESTING OF AGED OIL GRIMMER EXTRACTS AT 80% S-9 212
7.2.6 AMES TESTING OF SOLID PHASE EXTRACTS 212

7.3 DEVELOPMENT OF A NEW EXTRACTION METHOD FOR TESTING OIL PAH MUTAGENICITY 214
7.3.1 SOLID PHASE EXTRACTION (SPE) 215
7.3.2 NEW SORBENTS 216
7.3.3 THE C18/SILIA/ISOLUTE PAH HC METHOD 216

7.4 AMES TESTING OF NEW EXTRACTION METHOD 218
7.4.1 AMES TESTING OF BLACKBURN EXTRACTS WITH 80% S-9 218
7.4.2 AMES TESTING OF C18/SILICA/ISOLUTE PAH HC EXTRACTS WITH 80% S-9 219

7.4.2.1 C18/Silica/Isolute PAH HC Comparison to Grimmer, IP 346 and Blackburn Extracts....220
7.4.3 AMES TESTING OF AGED OIL BLACKBURN AND C18/SILICA/ISOLUTE PAH HC EXTRACTS....222
7.5 INHIBITION AND TOXIC EFFECTS FROM THE OIL AND OIL EXTRACTS....223
7.5.1 INHIBITION....223
7.5.1.1 Inhibition at 80% S-9....225
7.5.2 TOXICITY....225
7.6 TRENDS IN PAH CONTENT OF OILS 1-12....227
7.6.1 IMMUNOASSAY....227
7.6.2 COMPARING IP 346, IMMUNOASSAY AND C18/SILICA/ISOLUTE PAH HC EXTRACT DATA....227
7.6.3 IDENTIFICATION OF PAHS IN OIL....228
7.6.4 IDENTIFICATION AND QUANTIFICATION OF PAHS IN AGED OIL....230
7.6.5 MEASURING THE PAH CONTENT OF NON-TRANSFORMER OILS USING THE C18/SILICA/ISOLUTE PAH HC EXTRACTION....231
7.7 OVERALL CONCLUSIONS OF THE THESIS....232
7.8 FURTHER WORK....235
REFERENCES....238
APPENDICES....252

LIST OF FIGURES

FIGURE 1.1. Examples of PAH structures. 6

FIGURE 1.2. Bioactivation of Benzo[a]pyrene by mammalian liver enzymes. 8

FIGURE 1.3. Interaction of PAH epoxide and guanine on a DNA stand 9

FIGURE 1.4. The bay region of benzo[a]pyrene. 10

FIGURE 1.5. The principle of the Ames test.. 21

FIGURE 1.6. The principle components of a Gas Chromatogram (GC). 31

FIGURE 1.7. A GC slit/slitless injector. 31

FIGURE 1.8. The principle components of a Mass Spectrometer (MS 35

FIGURE 1.9. Outline of the competitive PAH immunoassay kit.. 36

FIGURE 2.1. Outline of the C18/Silica/Isolute extraction of PAHs from transformer Oil.. 55

FIGURE 3.1. Ames test revertants on the addition of chrysene 65

FIGURE 3.2. Ames test revertants on the addition of a mixture of the EPA 16 priority PAHs 66

FIGURE 3.3. Ames test revertants on the addition of liquid-liquid extracts of white oil 76

FIGURE 3.4. Ames test revertants on the addition of liquid-liquid extracts of oil 4.77

FIGURE 3.5. Ames test revertants on the addition of liquid-liquid extracts of oil Nytro-10GBN.....78

FIGURE 3.6. Ames test revertants on the addition of liquid-liquid extracts of oil 8.79

FIGURE 3.7. The total ion chromatograms (TIC) of oil 8 before extraction, after Grimmer extraction and after IP 346 extraction. 80

FIGURE 3.8. The Ames test revertants on the addition of Grimmer oil extracts 82

FIGURE 3.9. The Ames test revertants on the addition of Grimmer extracts of aged oils.....83

FIGURE 3.10. The Ames test revertants on the addition of Grimmer extracts of aged oil 84

FIGURE 3.11. Ames test revertants on the addition of two fractions of oil 4 large produced by scale solid phase extraction.....87

FIGURE 3.12. Ames test revertants on the addition of two fractions of oil Nytro-10GBN produced by scale solid phase extraction 88
FIGURE 3.13. Ames test revertants on the addition of two fractions of oil 8 produced by scale solid phase extraction.....89
FIGURE 3.14. Total ion chromatogram of the two PAH fractions of Nytro-10GBN from silica solid phase extraction 89
FIGURE 3.15. Ames test revertants on the addition of Nytro-10GBN Grimmer/SPE extracts 91
FIGURE 3.16. Total ion chromatogram of Nytro-10GBN fractionated by the Grimmer/SPE extraction.....92
FIGURE 4.1. The TIC of Nytro-10GBN silica solid phase extraction with methanol elution. 103
FIGURE 4.2. Spiked white oil elution efficiency of acetone compared to ethyl acetate with silica solid phase extraction. 104
FIGURE 4.3. TIC comparison of the cyclohexane washes and ethyl acetate eluent of Nytro-10GBN from columns using activated and inactivated silica 105
FIGURE 4.4. TIC of silica 10 mm x 460 mm column compared to a 20 mm x 200 mm column with Nytro-10GBN 107
FIGURE 4.5. Extraction efficiency of spiked white oil through a solid phase extraction with silica or alumina as the sorbent.....109
FIGURE 4.6. TIC spectra of Nytro-10GBN extracted with Al and Si SPE.....110
FIGURE 4.7. TIC of cyanopropyl/silica extraction 111
FIGURE 4.8. Extraction efficiency of spiked N10GBN oil through a cyanopropyl/silica column 112
FIGURE 4.9. TIC of oil Nytro-10GBN extracted with an Isolute PAH HC column using hexane (A) and cyclohexane (B) 113
FIGURE 4.10. Extraction efficiency of EPA 16 priority PAHs spiked into white oil using an Isolute PAH HC column.....114
FIGURE 4.11. TIC of oil 8, Nytro-10GBN and oil 4 using the mineral insulating oil fingerprinting technique.....117
FIGURE 4.12. TIC of Oil Nytro-10GBN removed from a C18 column with cyclohexane.....118

FIGURE 4.13. The TIC of oil 8 with Grimmer Liquid-liquid extraction compared to the liquid-liquid extraction in Wilson and Pahlavanpour (2000) prior to passage through an Isolute PAH HC column 120
FIGURE 4.14. The TIC of the liquid-liquid extraction in Wilson and Pahlavanpour (2000) followed by Isolute PAH HC. 120
FIGURE 4.15. The TIC of Grimmer liquid-liquid extraction followed by Isolute PAH HC 121
FIGURE 4.16. TIC of Nytro-10GBN from C18 clean-up followed by Isolute PAH HC purification 123
FIGURE 4.17. TIC of Nytro-10GBN clean-up with silica followed by C18 sorbent124
FIGURE 4.18. TIC of Nytro-10GBN clean-up with silica followed by C18 sorbent124
FIGURE 4.19. TIC Scan for N10GBN Transformer Oil spiked with 2 μgmL^{-1} extracted by C18 Silica PAH HC Isolute method.....126
FIGURE 4.20. PAH losses during the C18/Silica/Isolute PAH HC extraction process. 131
FIGURE 4.21. Extraction efficiency of phenanthrene, indeno[1,2,3-c,d]pyrene, benzo[b]fluoranthene and benzo[k]fluoranthene when added in a mixture with the EPA 16 priority PAHs at up to 4 $\mu g\ mL^{-1}$ per PAH. 133
FIGURE 5.1. The Ames test revertants on the addition of Blackburn oil extracts of white oil and oil 4 137
FIGURE 5.2. The Ames test revertants on the addition of Blackburn oil extracts of oil Nytro-10GBN and oil 8 139
FIGURE 5.3. TIC of oil 8 large scale C18/silica/Isolute PAH HC extract 143
FIGURE 5.4. TIC of oil Nytro-10GBN large scale C18/silica/Isolute PAH HC extract 143
FIGURE 5.5. TIC of oil 4 large scale C18/silica/Isolute PAH HC extract 144
FIGURE 5.6. TIC of white oil large scale C18/silica/Isolute PAH HC extract 144
FIGURE 5.7. Mutagenicity of the C18/silica/Isolute PAH HC extract of white oil and oil 4 146
FIGURE 5.8. Mutagenicity of the C18/silica/Isolute PAH HC extract of oil Nytro-10GBN and oil 8 148

FIGURE 5.9. The Ames test revertants on the addition of Blackburn oil extracts of aged oil without S-9 151
FIGURE 5.10. The Ames test revertants on the addition of Blackburn oil extracts of aged oil with 80% S-9 152
FIGURE 5.11. The Ames test revertants on the addition of C18/silica/Isolute PAH HC extracts of aged oils without S-9.....153
FIGURE 5.12. The Ames test revertants on the addition of C18/silica/Isolute PAH HC extracts of aged oil with 80% S-9 154
FIGURE 5.13. Toxicity effects of Grimmer extracts on *S. typhimurium*.....160
FIGURE 5.14. Toxicity effects of Blackburn extracts on *S. typhimurium*.....161
FIGURE 6.1. Phenanthrene solution prepared in-house to determine the accuracy of the total PAH kit within the range stated by the manufacturer 172
FIGURE 6.2. Benzo[a]pyrene solution prepared in-house to determine the accuracy of the carcinogenic PAH kit within the range stated by the manufacturer 173
FIGURE 6.3. Comparison of the percentage total and carcinogenic PAHs detected in transformer oils 1 to 12. 177
FIGURE 6.4. The TIC of oils extracted by the C18 Silica Isolute PAH HC method. 178
FIGURE 6.5. A close up of the first 17 minutes of the spectra shown in Figure 6.4179
FIGURE 6.6. IP 346, total immunoassay, GCMS TIC integration and GCMS SIC integration compared for transformer oil 1 to 12.....181
FIGURE 6.7. The TIC of oils ALT 0, ALT 1, ALT 2 and ALT 3 191
FIGURE 6.8. Area of the total ion chromatogram for aged.....192
FIGURE 6.9. Close up of diesel extracted by C18 Silica Isolute PAH HC method.196
FIGURE 6.10. Close up of petrol extracted by C18 Silica Isolute PAH HC method. 197
FIGURE 6.11. Close up of Sunflower Oil extracted by C18 Silica Isolute PAH HC method.....198
FIGURE 6.12. Close up of Olive Oil extracted by C18 Silica Isolute PAH HC method 199
FIGURE 7.1. Outline of work progression throughout this thesis to determine the mutagenicity of PAHs in transformer oil.....203

LIST OF TABLES

TABLE 1.1. IARC 15 carcinogenic PAHs 11

TABLE 1.2. EPA 16 priority PAHs.....11

TABLE 1.3 Solvent eluotropic values. 27

TABLE 2.1. Details of the transformer oil samples 1-12 and Nytro-10GBN 39

TABLE 2.2. The contents of the negative (non-mutagen) and positive (mutagen with 10% v/v S-9) control plates incorporated into top agar in the Ames test.. 45

TABLE 2.3. GC-MS retention windows for the selective ion chromatogram (SIC) of the EPA 16 priority PAHs.....53

TABLE 3.1. Effect of increased volume of DMSO as a solvent carrier in the Ames test 63

TABLE 3.2. Mutagenicity of PAH standards tested with the Ames test.....64

TABLE 3.3. Ames test revertants on the addition of pure oil 68

TABLE 3.4. Ames test revertants on the addition of oil 8 made soluble with Tween 80 69

TABLE 3.5. Effects on revertant number of adding whole oil to mutagens benzo[a]pyrene and 2-amino anthracene 70

TABLE 3.6. Effects of pure oil on S-9 activity and *S. typhimurium* growth.....73

TABLE 3.7. Percentage PAH extracted using the IP 346 and Grimmer methods 75

TABLE 3.8. Extraction efficiencies of large scale solid phase extraction with 20 mL and 10 mL of Nytro-10GBN oil.....86

TABLE 3.9. Effects on revertant number of adding Grimmer extracts to mutagens 93

TABLE 3.10. Effects on revertant number of adding solid phase extraction fractions 1 and 2 to mutagens 94

TABLE 3.11. Effects on revertant number of adding Grimmer/solid phase extraction fractions 1 and 2 to mutagens 95

TABLE 4.1. EPA 16 priority PAHs detected in white oil using GC-MS SIC quantification using Turbomass software 102

TABLE 4.2. Column dimensions investigated to determine the impact on extraction efficiency.....107
TABLE 4.3. Calibration data including slope deviation and limit of detection for quantification of the EPA 16 priority PAHs spiked into oil 127
TABLE 4.4. Extraction efficiency of spiked white oil (2 μg mL^{-1}) and unspiked oil through a small scale C18/silica/Isolute PAH HC. 128
TABLE 4.5. Extraction efficiency of spiked Nytro-10GBN oil (2 μg mL^{-1}) and unspiked oil through a small scale C18/silica/Isolute PAH HC. 129
TABLE 4.6. Percentage loss of PAHs throughout the extraction method and calculation of unaccounted species 132
TABLE 5.1. Miniscreen results for transformer oil Grimmer extracts, Brooks *et al.*, (1995).....140
TABLE 5.2. Miniscreen results for the Grimmer extracts of transformer oils, Burke *et al.*, (1996).....141
TABLE 5.3. Number of revertants on the addition of column residue from C18/silica clean up with Isolute PAH HC purification with and without 80% S-9 145
TABLE 5.4. Number of revertants on the addition of Nytro-10GBN extracted by C18/silica clean up with Isolute PAH HC purification at 580 mg $plate^{-1}$.....149
TABLE 5.5. Numbers of revertants on the addition of Nytro-10GBN extracted by 20 x small scale columns with C18/silica clean up with Isolute PAH HC purification. Total oil extracted was 5 g. 150
TABLE 5.6. Effects on revertant number of adding Blackburn extracts to mutagens benzo[a]pyrene and 2-amino anthracene (10% S-9).....155
TABLE 5.7. Effects on revertant number of adding C18/silica/Isolute PAH HC extracts to mutagens benzo[a]pyrene and 2-amino anthracene (10% S-9).....156
TABLE 5.8. Effects on revertant number of adding C18/silica/Isolute PAH HC extracts to mutagen 2-amino anthracene (80% S-9) 157
TABLE 5.9. The volumes of components in the *S. typhimurium* toxicity test compared to the Ames test.....159
TABLE 6.1. The affect of solvent conditions on the blank response of the PAH immunoassay.....171
TABLE 6.2. Calibration data for the total PAH immunoassay kit. 174

TABLE 6.3. Calibration data for the carcinogenic PAH immunoassay kit.....175
TABLE 6.4. Data for the total PAH immunoassay kit for transformer oils 1 to 12. 175
TABLE 6.5. Data for the carcinogenic PAH immunoassay kit for transformer oils 1 to 12.....176
TABLE 6.6. Identification of PAHs in Nytro-10GBN C18/Silica/Isolute PAH HC extracts compared to those found by the National Grid Company Plc.....183
TABLE 6.7. PAHs identified in oil 1 to 12 using the C18/Silica/Isolute PAH HC extraction method after analysis with GCMS TIC.....185
TABLE 6.8. Calibration data for the EPA 16 priority PAHs 187
TABLE 6.9. Amount of internal standard recovered after injection with oil extracts of oils 1 to 12.....188
TABLE 6.10. The amount of the EPA 16 priority PAHs found in 100 µL of transformer oil.....189
TABLE 6.11. Amount of internal standard recovered after injection with oil extracts of oils 1 to 12. 193
TABLE 6.12. The amount of the EPA 16 priority PAHs found in 100 µL of aged oils. 194
TABLE 7.1. Summary of Ames test results for SPE extracts. The mean number of revertants is shown.....213
TABLE 7.2. Compiled results of the Ames data for all aromatic extracts. 222

LIST OF PLATES

PLATE 1.1. Colony revertants on negative and positive control plates in the Ames test. 22

PLATE 1.2. SPE cartridges for solid phase extraction. 26

LIST OF APPENDICES

APPENDIX A: ADDITIONAL MATERIALS 253

APPENDIX B: ADDITIONAL METHODS 256

APPENDIX C: THE AMES TEST RAW DATA 259

APPENDIX D: ION CHROMATOGRAM PEAK AREA FOR ESTIMATING TOTAL PAH CONTENT 263

NOTATION

% v/v	Percentage volume for volume
% w/v	Percentage weight for volume
% w/w	Percentage weight for weight
%CV	Coefficient of variance
2-aa	2-amino anthracene
Al_2O_3	Alumina
B[a]P	Benzo[a]pyrene
DMSO	Dimethyl sulphoxide
EI	Electron Ionisation
EPA	Environmental Protection Agency (USA)
EPA 16 PAHS	EPA 16 Priority PAHs
GC	Gas Chromatography
HPLC	High Pressure Liquid Chromatography
LLE	Liquid-Liquid Extraction
min	Minutes
MS	Mass Spectroscopy
n	Number of samples
NaCl	Sodium Chloride
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NIST	National Institute of Standards and Technolgy
OECD	Organisation for Economic Co-operation and Development
PAH	Polycyclic Aromatic Hydrocarbons
S-9	Rat liver homogenate
SD	Standard Deviation
SiO_2	Silica gel
SIC	Selective Ion Chromatogram
SPE	Solid Phase Extraction
TIC	Total Ion Chromatogram
UV	Ultra Violet

CHAPTER 1.0

INTRODUCTION AND LITERATURE REVIEW

1.1 TRANSFORMERS AND TRANSFORMER OIL

Electricity is transferred from the source of production to homes and industries at high voltage. This is to avoid losses in power due to the resistance that occurs over long distances. Transfer across the country is via cables, which are situated either underground or overhead. In order to reduce the danger from high voltages as the electricity gets closer to residential areas, the voltage is minimised. This is achieved with the use of a transformer. To minimise heat loss from transformers and to stop conduction, a variety of mineral oils are used. The oil must allow the transformer to withstand lightning, over-voltage and overload stresses. The oil also prevents water entering the transformer, therefore reducing corrosion. Up to 100 000 litres of oil is required to fill a transformer and the quality of the oil is crucial in determining the efficiency and lifetime of a transformer (National Grid Company Plc, 2002).

1.1.1 Composition of Transformer Oil

The mineral oils most commonly used in transformers are naphthenic, which refers to the high contents of cyclic compounds present including aromatic compounds such as polycyclic aromatic hydrocarbons (PAHs). The oil also contains minor components such as oxygen, sulphur, nitrogen, metals and water, which vary according to the degree of refining performed. As the working life of a transformer is so dependent on the oil's properties, the main problems that occur in a transformer are related to the inconsistent nature of the oil. Grades of oil have been engineered according to the amount of power that will pass through the transformer. This is not the only way that composition will vary however, as it will also alter with the origins of the crude oil raw material used (Nynas, 1999).

Oil has many functions in a transformer. In addition to reducing the stress from changes in voltage, it can indicate the condition of the transformer if measured according to strict standards. The colour and general appearance of the oil are characteristics that can be assessed by an experienced person. For example, a

darkening of the oil indicates deterioration, while debris in the oil might suggest breakdown of fibres or cellulose from insulation paper in the transformer. Water is also an indication of problems in the transformer. The water may originate from the insulation paper or from the oxidation of the oil and leads to more rapid decomposition. Furthermore, oxidation leads to increased acidity which can have a corrosive effect on the transformer (Nynas, 2001)

Transformer oil has now been used for a 100 years, but ensuring dryness and reducing air contact during the lifetime of the transformer is a problem that keeps the industry searching for alternatives to oil insulation. As the oil works so effectively however, alternatives that are as economically viable are unlikely to be found in the near future (Lindroth, 1995).

1.1.2 The Breakdown of Oil

One problem with transformer oil is that it readily breaks down in the presence of 0.05-0.25% oxygen. Even with degassing processes the oil naturally contains this small amount of oxygen. Heat, UV light, mechanical shear or high electrical fields will cause the production of free radicals that will react with oxygen and produce further radicals which are unstable. In this way the chain reaction of oxidation is perpetuated (Nynas, 2001).

PAHs decompose peroxide into stable compounds and absorb hydrogen gas (produced by electric discharges in the oil) by taking up hydrogen on the unsaturated aromatic rings and therefore stopping more free radicals forming (Nynas, 2001). In this way, PAHs are natural inhibitors in the oil, although oils that contain only naturally occurring inhibitors are classed as non-inhibited. Inhibited oils are those that have phenol added in addition, which further stabilises the oil by donating a hydrogen to free radicals produced during oxidation. Most oils have an inhibitor added at various quantities, but naturally occurring PAHs remain important in prolonging the lifetime of the oil.

1.1.3 Carcinogenicity of the Oil

Epidemiological studies for many years have identified links between oils and cancer, which is believed to be the primary hazard from exposure to oil (Järvholm and Easton, 1990; Hewstone, 1994). These studies looked at a number of different occupations where workers were commonly exposed to mineral oils. Particular links were made with skin and bladder cancer, but other cancers of the stomach, colon, rectum, pancreas, larynx and prostate were also observed (Tolbert P.E, 1997). The type of exposure played an important role in which cancers resulted, although such studies draw no conclusive classification.

1.1.3.1 PAH Mutagenicity Versus Aromatic Mutagenicity

The mutagenicity of oil is attributed to the polyaromatic (PAH) fraction of the oil (McKee *et al.,* 1989; Järvholm and Easton, 1990; Brooks *et al.,* 1995; Granella *et al.,* 1995) as PAHs are one of the few known mutagens to have been identified in the oil (Grimmer, 1983). Oils that have had the polyaromatic content extracted by polar solvents have been shown to lose their carcinogenic properties (IARC, 2002).

The oil industry uses a liquid-liquid extraction called the IP 346 method (BS2000 Part 346, 1996) (Section 1.5.2) to measure the carcinogenic threat of polyaromatics from the oil. The method uses the weight of the oil before and after extraction to give a percentage value of polyaromatic content. However, the IP 346 % w/w method (and other liquid-liquid extractions) is known to remove all oil components soluble in dimethyl sulphoxide (DMSO) which may include simple aromatics and other polar components. Therefore the IP346 % w/w method, in addition to alternative liquid-liquid extractions involving DMSO, over estimates polyaromatic content (Stang, 1993; 1999). For this reason the fraction of polyaromatic and aromatic compounds extracted by liquid-liquid extractions will be referred to as the aromatic content, although it is the polyaromatics (PAHs) that is estimated by these methods.

Nevertheless the IP 346 % w/w method is used to measure carcinogenic risk from the oils, as IP 346 % w/w data correlates with skin painting experiments and this carcinogenicity is attributed to PAHs (Grimmer, 1983). An IP 346 aromatic fraction of more than 3% has been chosen by the Oil Companies' European Organisation for Environmental and Health Protection (CONCAWE) as oils containing a lower IP 346 content showed no carcinogenicity (Nynas, 1999). Oils that contain more than 3% must be labelled as hazardous (Jansson, 1992; Hewstone, 1994; Granella *et al.,* 1995).

Such concerns over the health impacts of PAHs could be removed by refining the oil further thus removing the PAH content. However, removing the PAHs without extracting other aromatic components of the oil is difficult and such refining would change the properties of the oil, making it unsuitable for use in transformers. It would also increase the requirement for artificial inhibitors like phenol, which is corrosive, mutagenic and highly toxic when heated. For this reason, the National Grid Company Plc requested a mutagenic study of PAHs in transformer oil, which forms the basis of this work.

1.2 POLYCYCLIC AROMATIC HYDROCARBONS

Polycyclic aromatic hydrocarbons (PAHs) are found naturally in oils but they are also commonly found in the open environment, as the combustion or pyrolysis of almost all organic compounds will lead to small amounts of PAHs being produced (Moret and Conte, 2000, Wang *et al.,* 2000). Alternative sources therefore include the combustion of fossil fuels, vehicle emissions and cigarette smoke. Of these sources Grimmer *et al.,* (1991) states that over 75% of the carcinogenicity seen in animal experiments was due to PAHs with 4 rings or more. PAHs are so hazardous that they have been included by the Environmental Protection Agency (EPA) in the USA on the list of priority pollutants (EPA, 2002).

Polycyclic aromatic hydrocarbons have been given many names including polyarenes, polyaromatic compounds and polynuclear aromatic hydrocarbons. For the purpose of this work, these molecular structures will be mainly referred to as PAHs or polyaromatics when discussing a mixture of unknowns, such as that extracted by liquid-liquid methods.

1.2.1 PAH Structure

PAHs are based on benzene rings, which are unsaturated six-membered hydrocarbon rings (Figure 1.1). PAHs, like benzene are far more stable than the alkenes although the presence of double bonds would suggest that it should be similar. The explanation for this extra stability lies in the distribution of electrons in the molecular orbitals of the cyclic structure. All six carbons are sp^2 hybridised and all carbons have equivalent p-orbitals perpendicular to the plane of the ring. These p-orbitals overlap with neighbouring p-orbitals, becoming degenerate and therefore more stable, forming a cloud of electrons above and below the plane of the carbon ring. If a molecule exhibits this characteristic, it is considered aromatic (Fessenden and Fessenden, 1994).

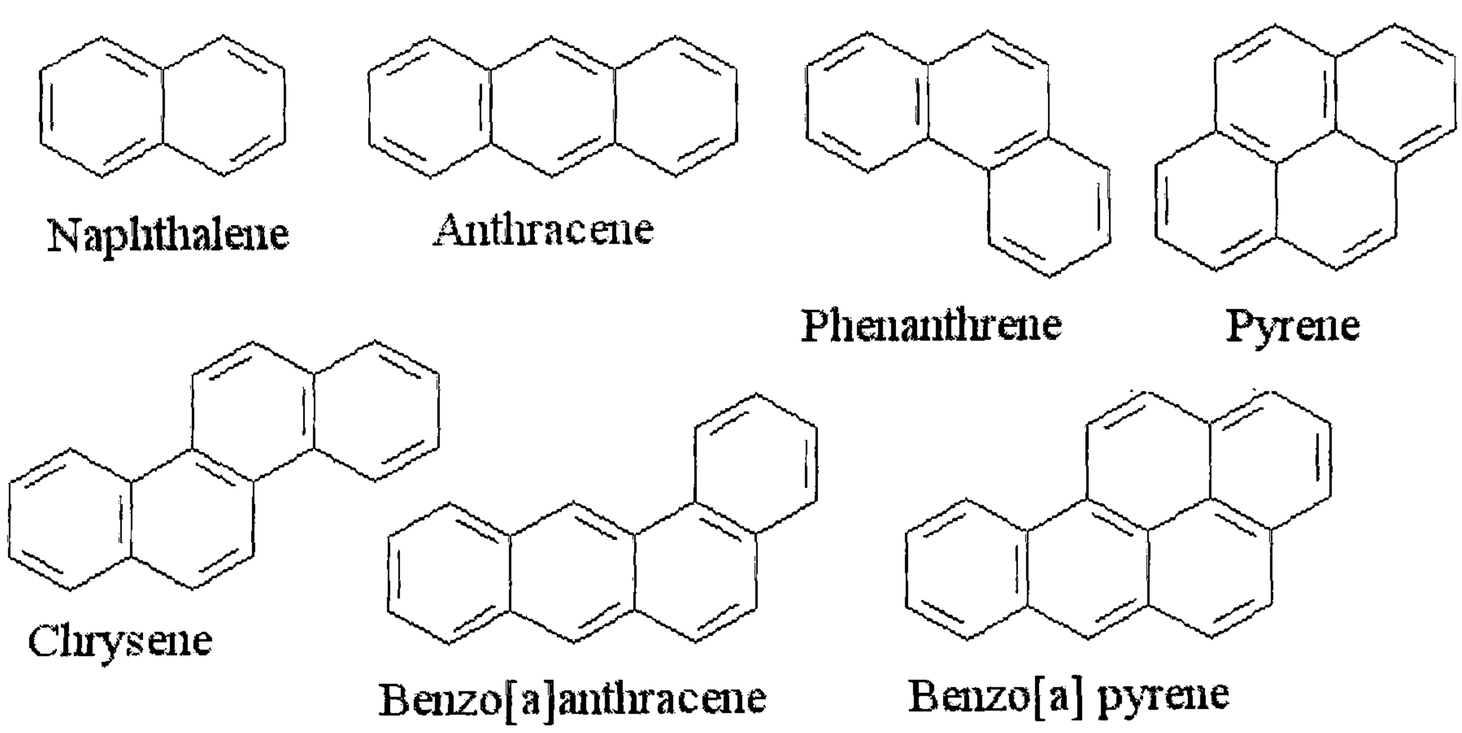


FIGURE 1.1. Examples of PAH structures.

1.2.2 Carcinogenic and Mutagenic PAHs

Carcinogens are chemical agents that act on the body and cause cancer. Carcinogenicity is irreversible; the repair mechanisms of the body may reverse some of the effects but this is not well understood and is believed not to be significant (Alberts *et al.*, 1989).

It has been determined that cancers can be initiated by a change in DNA sequence called a mutation. A mutation does not always lead to a cancerous tumour growth however, as cancer is usually the result of a number of independent incidents in a cell, which have a cumulative effect (Alberts *et al.,* 1989). PAHs are mutagenic initiators of cancer (Yuspa and Bethesda, 1986). They bind to the DNA in an activated form (Section 1.2.3) which disrupts the strand, and causes an altered transcription of the strand. Once the cells have been exposed to a tumour promoter above a threshold level, tumour production can take place no matter how much time has passed since exposure to the mutagenic initiator (Alberts, *et al.,* 1989).

Links between cancer and hydrocarbons were first observed in 1775 when scrotal cancer in chimney sweeps was linked to occupational exposure to soot. It was not until 1915 however, that experimentation in Japan provided direct evidence of hydrocarbon carcinogenicity by applying coal tar directly to the skin of animals, which was followed by the identification of specific carcinogenic PAHs (Harvey, 1991). The 3 to 7 ring PAHs in mineral oil are believed to be the source of carcinogenicity in mouse skin painting assays (Roy *et al.,* 1996).

1.2.3 PAH Activation

PAHs must be activated before they can demonstrate mutagenic activity and are therefore known as indirect mutagens (as opposed to direct mutagens that do not require activation). Activation occurs in mammals as a method of making xenobiotics more soluble and therefore facilitating removal (excretion) from the body. Metabolic

activation occurs in two phases. Phase I uses a multi-step pathway involving cytochrome P450; particularly form 1A1 (Bauer *et al.,* 1995) to introduce a functional group such as a hydroxyl group. Phase II reactions connect this functional group to an endogenous species (e.g. glucuronic acid) to create a hydrophilic molecule that can be easily excreted. In the case of many xenobiotics, including PAHs, metabolic intermediates produced after phase I are mutagenic.

The phase I cytochrome P450 oxidises the PAH to an epoxide and then an epoxide hydrase converts this to a dihydrodiol. This is then oxidised by another P450 and produces the dihydrodiol epoxide that will react with DNA (Figure 1.2) (Josephy *et al.,* 1997).

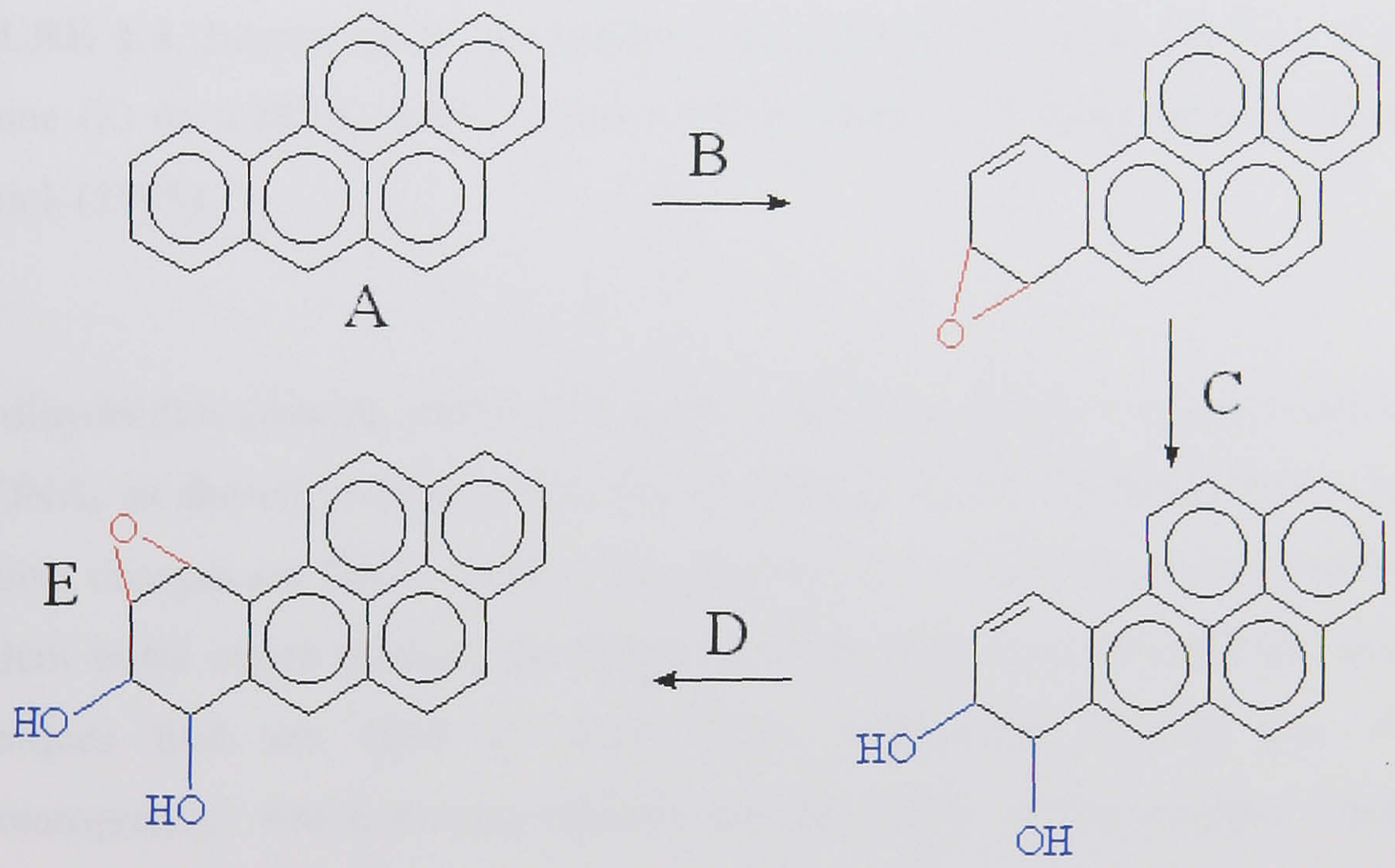


FIGURE 1.2. Bioactivation of Benzo[a]pyrene by mammalian liver enzymes Adapted from Weaver and Hedrick (1995). Benzo[a]pyrene (A) is an epoxide by cytochrome P450 (B) and then an epoxide hydrase (C) converts it to a dihydrodiol. This is then oxidised by another P450 (D) and produces the dihydrodiol epoxide (E) that will react with DNA.

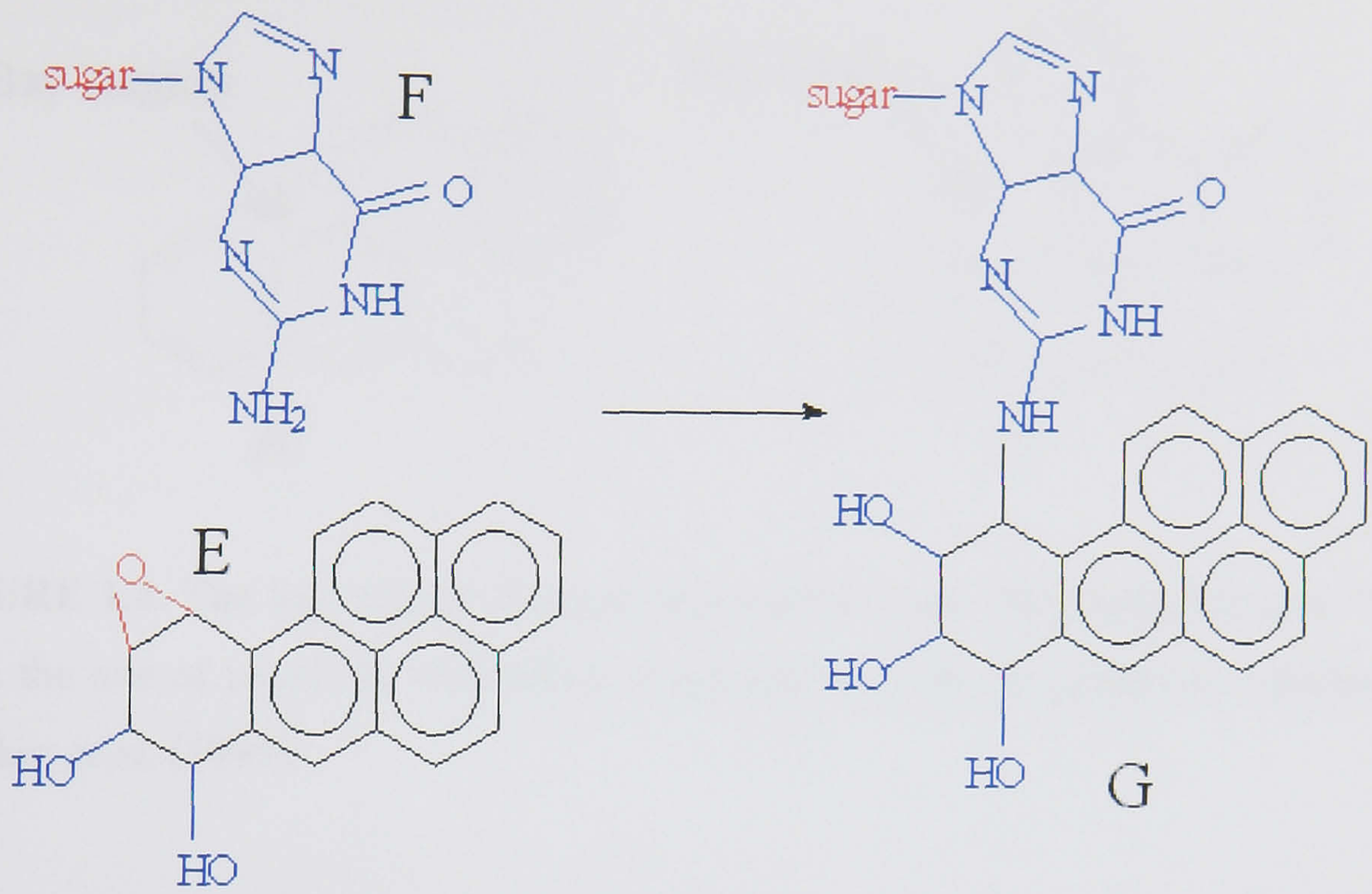


FIGURE 1.3. Interaction of an carcinogenic epoxide (E) from Figure 1.2 with and guanine (F) on a DNA stand to form a DNA adduct (G). Adapted from Weaver and Hedrick (1995).

The dihydrodiol epoxide shown in Figure 1.2 has the ability to interact with bases in the DNA, as shown in Figure 1.3. The epoxide on the PAH metabolite is drawn to negative charges on DNA, such as the electron rich centre of guanine, and forms a covalent bond which disrupts the DNA structure (Weaver and Hedrick, 1995). The techniques that are used to study DNA disruptions include gas or liquid chromatography, which detects adducts and as PAHs are fluorescent, fluorescence spectroscopy may also be used (Lodovoci *et al.,* 1998).

A structural indication of mutagenicity has been established and studied. When a diol epoxide is formed in the bay region of a PAH (shown in Figure 1.4) containing 4 to 5 rings, the resulting structure is likely to be a mutagen (Buening *et al.,* 1979; Glucker *et al.,* 1999). When a methyl group is present in the bay region, many PAHs will exhibit an enhancement of mutagenicity (Harvey, 1991). However, the presence of the bay region does not indicate that a PAH will be carcinogenic (Zeiger, 2001).

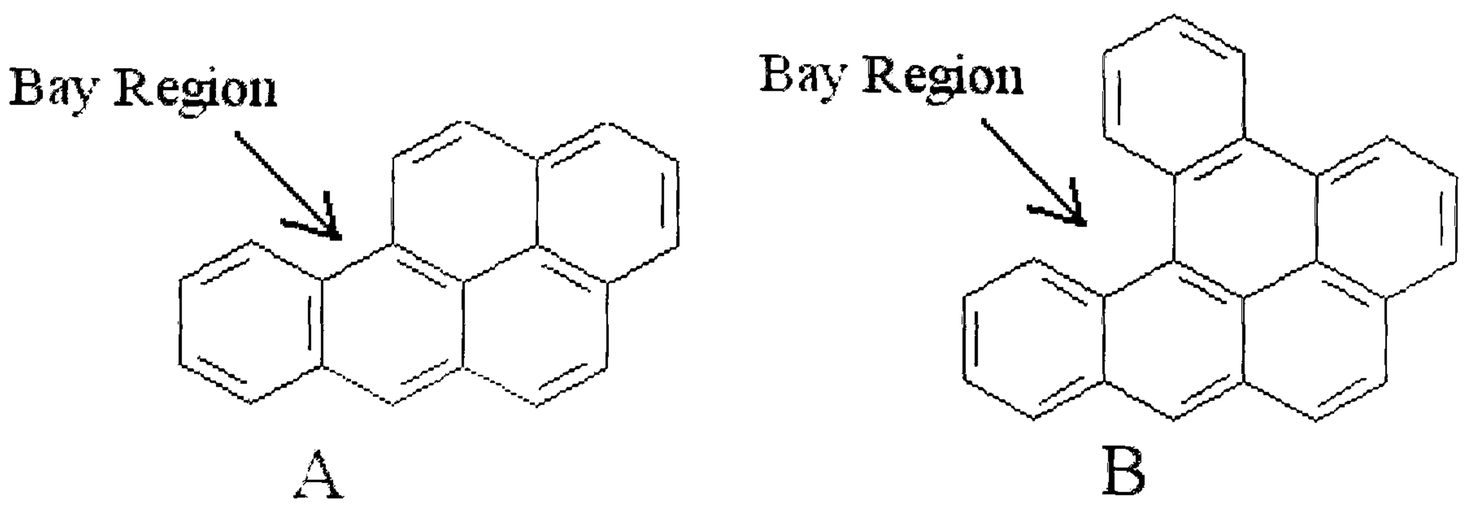


FIGURE 1.4. The bay region of benzo[a]pyrene (A) and dibenzo[a,i]pyrene, thought to be the site of reactivity with DNA when activated by oxygenation. Adapted from Glucker *et al.* (1999).

1.2.4 IARC Classification of PAHS

Not all PAHs are thought to be carcinogenic, and of those that are, some are more carcinogenic than others. The International Agency for Research on Cancer (IARC, 2002) has reviewed all the literature available on PAHs and has created a classification of carcinogenicity which is as follows:

Class 1: Carcinogenic to humans
Class 2A: Probably carcinogenic to humans
Class 2B: Possibly carcinogenic to humans
Class 3: Not classifiable
Class 4: Probably not carcinogenic to humans

In addition to this classification system, there are three degrees of evidence that give rise to these classifications; sufficient, limited and inadequate. Such general classification, in addition to differing levels of evidence, makes it difficult to establish any definite conclusions (Hewstone, 1994).

Mineral oils are defined in classes according to refinement (Bingham, 1988). Unrefined mineral oils fall into Class 1, whereas mildly refined oils are considered 2A or 2B. With no definition of what 'mildly refined' is, it is difficult to use such a classification. Highly refined oils that contain little or no PAHs are considered class 4, but it must be noted that they are classed as such due to inadequate data on carcinogenicity. There are 15 PAHs that are classed as having sufficient evidence of carcinogenicity in animals and they are listed in Table 1.1.

TABLE 1.1. IARC 15 carcinogenic PAHs.

PAHs Classed as Having Sufficient Evidence for Carcinogenicity by the IARC		
Benz[a]anthracene	7-H-Dibenzo[c, g]carbazole	Indenol[1, 2, 3-cd]pyrene
Benzo[b]fluoranthene	Dibenzo[a, e]pyrene	5-Methylchrysene
Benzo[j]fluoranthene	Dibenzo[a, h]pyrene	Dibenzo[a, h]acridine
Benzo[k]fluoranthene	Dibenzo[a, i]pyrene	Dibenzo[a, j]acridine
Benzo[b]pyrene	Dibenzo[a, i]pyrene	Dibenzo[a, h]anthracene

TABLE 1.2. EPA 16 priority PAHs.

PAHs Classed as Priority Pollutants by the EPA		
Naphthalene	Fluoranthene	Benzo[a]pyrene
Acenaphthylene	Pyrene	Indeno[1,2,3-c,d]pyrene
Acenaphthene	Benzo[a]anthracene	Dibenz[a,h]anthracene
Fluorene	Chrysene	Benzo[g,h,i]perylene
Phenanthrene	Benzo[b]fluoranthene	
Anthracene	Benzo[k]fluoranthene	

Some of these PAHs have been included on the EPA's list of 16 PAH priority pollutants (EPA, 2002), in addition to non-mutagenic PAHs that are hazardous due to their abundance in the environment. These are listed in Table 1.2.

1.3 TESTS FOR CARCINOGENICITY

The first tests used to identify carcinogenic behaviour of chemicals were performed on mammals because they are most closely related to humans. Almost all forms of cancer found in humans can be reproduced in an animal model by the use of chemical carcinogens and in some cases, the additional application of a tumour promoter. All the major organs of the animal are exposed in these experiments to determine where the greatest damage is caused. The results of any animal test however, will differ according to many factors so it is important to reduce as many variables as possible. These factors may be animal specific such as, species, age, gender or diet. It may also depend on the techniques used, such as, solvent selection, hair removal method, amount and concentration of each dose, frequency and duration of treatment or which end point is employed (Grimmer, 1983).

PAHs are of most concern to the general public through contamination of food (Phillips, 1999) so most experimental animals are exposed to PAHs orally. However, skin carcinogenicity is the most relevant type of testing to companies such as the National Grid Company Plc as this is the most likely means of employee exposure to oil. Skin is one of the easiest models to study as it is clearly visible and resulting local changes can be recorded using photographs (Grimmer, 1983). The drawback to this type of testing is that the skin is not uniform in its composition and may contain higher levels of enzymes in certain areas. As PAHs require metabolism before they have effect, the potency of carcinogenicity will differ depending on which part of the skin is used. Skin tests will also be affected by the condition of the skin (Grimmer, 1983).

As the skin provides a barrier between the body and the outside world, much of the carcinogen will remain on the skin surface and cause tumours locally. However, subcutaneous injections have shown that without such a barrier, the tumours are still mainly observed on the skin, illustrating that PAHs are local carcinogens (Grimmer, 1983). Each different site of administration will give different rates of metabolism and elimination from the body. However, as all types of delivery will lead to the

elimination of PAHs via the liver and gall bladder, all administration has the potential to cause tumours in these organs (Grimmer, 1983).

Although animal models are the closest to humans, it can take time for the cancer to develop and therefore great expense is incurred for the up-keep of the animals. Mendelsohn quoted the price of an animal test in 1988 to be $1 million per chemical tested. In addition, cancer is commonly a result of several types of exposures, so quantifying the carcinogenic effect of one particular substance is extremely difficult even under controlled conditions (Boffetta, 1993). Often cancer is subject to the susceptibility of the patient in terms of their genetic make up, which is difficult to quantify (Alberts, *et al.*, 1989).

1.3.1 Mutagenicity as a Measure of Carcinogenicity

Due to the expensive and time consuming nature of animal tests, as well as the associated ethical debate, bacteria offer an alternative to animals. However, bacterial tests, such as the Ames test (Section 1.5.1) have two drawbacks. The first is that bacteria are prokaryotic cells that do not relate well to human eukaryotic cells. The second is that a bacterial test detects the mutagenic properties of a chemical, which does not always correlate with carcinogenicity (Watson *et al.*, 1985; Zeiger, 2001). The literature often provides conflicting correlations between mutagenicity and carcinogenicity. A 91% correlation between carcinogenicity and mutagenicity was observed by Purchase *et al.* (1978) using 58 carcinogens and 62 non-carcinogens whereas Andrews *et al.* (1978) identified a correlation of only 58% when testing 25 PAHs.

Studies have been conducted to test the mutagenicity of 54 PAHs (Coombs *et al.*, 1976). Carcinogenicity data had already been made available for these PAHs, some from skin painting experiments on mice and others from injection experiments on rats, the results of which were compared with the bacterial data. Thirty-seven of the PAHs

were carcinogenic in animal experiments while all 54 exhibited mutagenic activity. In some cases the weakest carcinogens proved to be the most mutagenic.

Although this suggests that a measure of mutagenicity cannot determine if exposure to a chemical will lead to cancer, mutagenicity can still illustrate a hazardous property of a chemical. Mutagenicity, unlike carcinogenicity can be measured directly and rapidly as it is not a multi-step process and does not have a long latency period. It can indicate the ability of a chemical to initiate cancer, so is a valid measurement to investigate as a screening process for potential carcinogens. It will not of course detect non-mutagenic carcinogens (Haroun and Ames, 1981) but as PAHs have been identified as mutagenic, their impact in oil carcinogenicity can be measured with a mutagenicity test. For this reason, bacterial mutagenicity testing was used in this work.

1.3.2 PAH Toxicity

Like most substances, PAHs are toxic when in a large enough dose (Wong and Wang, 2001). Toxic effects can be split into two types, cytostatic and cytocidal toxicity. While cytocidal agents are lethal, cytostatic agents inhibit and slow growth rather than kill the cell.

Toxicity in addition to carcinogenicity has been difficult to assess due to the problems associated with the extrapolation of toxicity data from other species to humans. For this reason it is often not clear, particularly in mixtures, what level of PAHs constitute a threat to human health (Moret and Conte, 2000). PAH carcinogenicity is of greater concern in oil than toxicity, as carcinogenicity will generally be of threat before toxic effects are observed (Haroun and Ames, 1981). However, if any PAH shows great toxicity, it is possible that it would effect mutagenicity testing by killing or inhibiting the bacteria.

Cytocidal or cytostatic toxicity may be visible in mutagenicity tests by a reduction or complete absence of bacteria (such as the absence of a background bacterial lawn). However, such toxicity if not acute, may not be as visible and may mask mutagenic

effects by reducing the number of viable cells that can be mutated. This would lead to reduced numbers of revertants, even in the presence of a highly mutagenic chemical. If toxicity were found to significantly reduce the number of viable cells, a ratio of the viable cell number to revertant number would have to be measured to determine the reversion frequency. For example, if the number of revertants was reduced by 50% on plate 2 compared to plate 1, but the viable cell count on plate 2 showed 50% less cells present, the mutagenicity on plate 1 and 2 would be equivalent. Toxicity will be investigated therefore by growing the *S. typhimurium* culture with the test samples in nutrient growth to establish whether the test chemical has toxic properties that reduce the number of viable cells and therefore affects Ames testing (Section 5.8).

1.4 AIMS OF THESIS

Many studies have been performed investigating the PAH mutagenicity of oils (Herman *et al.,* 1981; Blackburn *et al.,* 1984, 1986; Ingram *et al.,* 1994; Granella *et al.,* 1991, 1995; Brooks *et al.,* 1995). The majority of oil mutagenicity testing centres on a liquid-liquid extraction step that recovers the aromatic fraction from the oil. Transformer oils contain a highly complex mixture of aromatics including PAHs, some of which may be mutagenic. Studying the mutagenicity of such extracts simplified the oil matrix, but attributing the mutagenicity of such extracts solely to PAHs was not possible. As the main concern for transformer oil hazard labelling legislation by CONCAWE was PAH mutagenicity, the work here centres on the isolation and measurement of PAH mutagenicity.

The aims of the work were as follows:

- Establish the extent to which PAHs contribute to total oil mutagenicity and therefore the risk they present to National Grid Company Plc employees. The mutagenicity of PAHs were compared to the mutagenicity of the aromatic fraction of the oil (extracted by liquid-liquid extractions) and the whole oil. This was to establish if the removal of PAHs from the oil removed the mutagenic threat significantly enough to warrant the loss of beneficial properties (reducing oil

breakdown) from the oil. Mutagenicity testing must include an investigation into possible antagonistic or synergistic effects that could interfere with measurements, including toxicity.

- Identification and quantification of the main PAHs present in the oil, particularly PAHs classed as priority pollutants by the Environmental Protection Agency (EPA). This involved the development of an extraction method to isolate PAHs from the rest of the oil matrix.

- Relate carcinogenic PAH content to total PAH content to determine if measuring total PAH (as in the IP 346 % w/w method) is a valid method of determining carcinogenic risk. This involved the use of immunoassay kits that are available for measuring both total PAH content and carcinogenic PAH content.

- Investigation of possible changes in PAH composition (and therefore PAH mutagenicity) with oil ageing. PAH composition is believed to increase with usage (Wong and Wang, 2001) which would improve oil oxidation stability but may increase oil mutagenicity and therefore increase the threat to the end-user over time. As used oils were not available for testing, artificially aged oil was tested for mutagenicity and PAH content as a measure of oxidation stability and health risk changes in used oil.

1.4.1. Constraints of Work

There were a number of considerations that controlled the manner in which the work was performed. The main factors that were noted at the beginning of the work are briefly considered here.

1.4.1.1 The End-User's Requirements

As the National Grid Company Plc and indeed any other concerned end-user may wish to repeat this work for new oils coming onto the market, it was specified that all methods used should minimise the financial cost of testing and the risk to the health of the user. This included substituting solvents for less hazardous ones. This was exemplified by the move towards a solid phase extraction method for sample preparation, as opposed to the current liquid-liquid extraction method, in order to reduced solvent consumption. This consideration was also a prime reason as to why the Ames test was the most appropriate choice for mutagenicity testing, rather than other commercially available tests (discussed further in Section 1.5.1.3). The Ames test can be set up easily and does not require expensive equipment. The strain of *Salmonella typhimurium* can be used on the bench and does not provided risk to health if handled correctly.

1.4.1.2 Oil Extracts for Both PAH Mutagenicity and Analysis

Although many methods are available in the literature for analysing PAHs in oil, such as chromatographic techniques or supercritical fluid extraction (section 1.5.3.4), they do not produce a sufficient mass of extract to allow mutagenicity testing. Furthermore, current liquid-liquid extraction methods used to measure oil mutagenicity are not amenable to quantitative analysis, as they extract total aromatic content rather than PAHs solely and are therefore too complex. The same extraction method should be used for mutagenicity testing and analysis in order to conclude that the identified PAHs are present in the extracts tested for mutagenicity. For this reason, liquid–liquid and solid phase extraction methods was developed, as they produce extracts in sufficient quantity to allow both analysis and mutagenicity testing.

1.4.1.3 Regulatory Concerns

In order to meet the regulatory standards governing the Ames test, four strains of *S. typhimurium* (TA98, TA100, TA1535 and TA1537) should be used to test the samples (Gollapudi and Krishna, 2000). However, this work was concerned with the review and development of improved ways to determine PAH mutagenicity, and is not required to meet such regulatory standards. For this reason only TA98 (shown to be the most susceptible strain to PAH mutagenicity by Hermann *et al,* (1980)) was used.

1.4.1.4 Variation in PAH Content with Oil Origin and Age

For users of transformer oils, the problem of determining the risk arising from PAHs is complicated by the fact that there is no guarantee that all oils will have a similar PAH content and composition. Gases produced as a result of oil deterioration during in-service usage react with naturally occurring PAHs and other components of the oil to alter the PAH composition (Moret and Conte, 2000). This is further complicated should the oil be mixed with those from another source and can have detrimental effects on transformer performance (Nynas, 2001). For these reasons, it is also highly desirable to find a way of monitoring the PAH composition of in-service oil that is quick and cheap. Ideally, an analysis method that is easy to perform on site rather than in the laboratory where testing is currently slow and requires large, expensive equipment and trained staff, would be beneficial.

1.4.1.5 PAH Mixtures in a Complex Matrix

The initial problem that occurs when beginning mutagenicity testing on PAH species in oil is that there is no agreement in the literature as to the mutagenic effect of PAHs mixtures. It is often assumed that toxic substances will show the sum of their toxic effects when in a mixture (White, 2002). This cannot always be true due to the synergistic and antagonistic effects known to occur in any chemical mixtures

(Manahan, 1994). This has led to a limited understanding of the underlying biological effects of toxic mixtures (Randerath *et al.,* 1999). Information concerning the mutagenic effects of PAH mixtures upon animals has been gathered, but shows widely differing results according to the route of exposure (Schneider *et al.,* 2002).

The mixture of PAHs that have been identified in oil (Pahlavanpour and Wilson, 1999) will have antagonistic and synergistic effects, which may increase or decrease the risk from PAHs in complex oil samples. Other components in the complex oil matrix are also likely to have antagonistic and synergistic effects, which must be investigated. These interferences may also have their own mutagenic properties, which ideally must be subtracted from the PAH mutagenicity in order to determine if PAHs present the greater risk to human health.

1.4.1.6 Volume of Oil for Testing

Another consideration when testing oil for mutagenicity was the quantity of oil to use. The carcinogenic PAHs may only make up a small percentage of the oil, but as 100 000 litres of oil are used in a single transformer, the risk becomes proportionally significant (National Grid Company Plc, 2002). For this reason, it is not unreasonable to suggest that the quantity of oil tested may have a significant impact on the final conclusion of the study.

As there are limits to the amount of oil that can be immobilised on the surface of an agar plate with the bacteria, PAHs will have to be concentrated to allow greater doses to be tested. The polyaromatic content of the oils provided by the National Grid Company Plc varies from 1-9% according to the IP346 % w/w method (Table 2.1) suggesting that a range of doses must be tested. The literature suggests typical oil volumes of 2-6 mL for subsequent Ames testing is sufficient to elicit a mutagenic response (Herman *et al.,* 1981; Blackburn *et al.,* 1984, 1986; Ingram *et al.,* 1994; Granella *et al.,* 1991, 1995; Brooks *et al.,* 1995). An oil volume of up to 6 mL will mix relatively well with 1 mL DMSO, a minimum volume required for conventional petri

dish based mutagenicity testing. When adding the extract of more than a 6 mL volume of oil to 1mL DMSO, the sample is not completely soluble. In addition, extracting more than 6 mL becomes more hazardous, as it requires more solvent to be used during the extraction method, which is detrimental to the end-user.

1.5 INTRODUCTION TO METHODOLOGIES

The following section provides an introduction to the methods used in this work. This includes the Ames test which is used for mutagenicity testing, liquid-liquid and solid phase extraction methods used to determine the PAH content of the oils and immunoassay and GCMS employed to detect and analyse the PAH composition.

1.5.1 The Ames Test for Mutagenicity

In the 1970s, Bruce Ames developed a test utilising *Salmonella typhimurium* that has become a standard test for the preliminary screening of potentially carcinogenic chemicals (Ames *et al.,* 1975).

The Ames test consists of a specially constructed *Salmonella typhimurium* auxotrophic histidine mutant strain incorporated into top agar and grown on a minimal glucose agar plate. Each mutant strain contains an alteration in one of the 8 genes of the histidine operon. With each mutation an enzyme will be inactivated, leading to incomplete histidine production so that only a trace of histidine present in the top agar keeps the bacteria alive. More detail is given in Section 1.5.1.2.

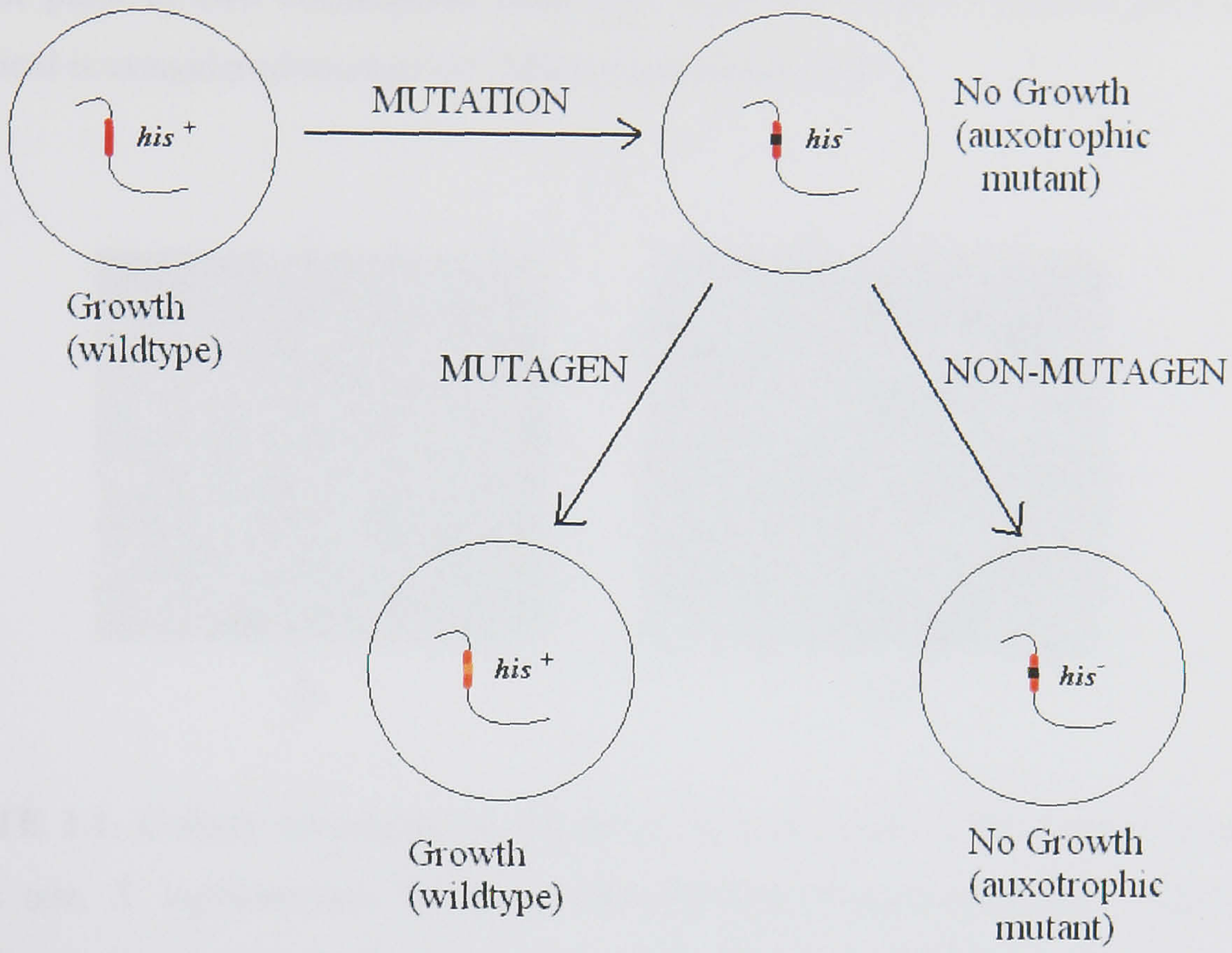
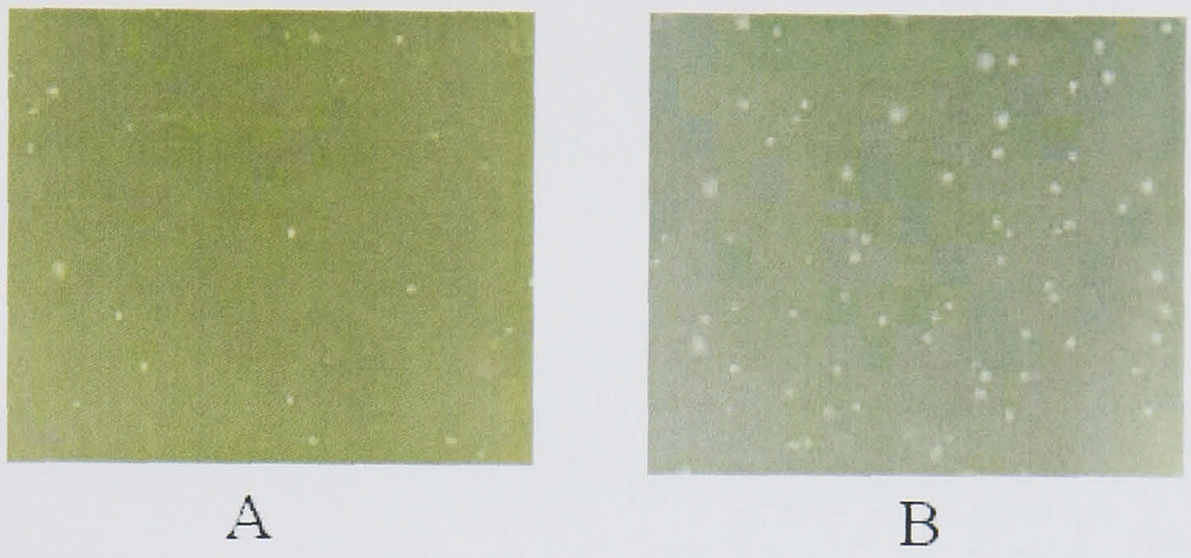


FIGURE 1.5. The principle of the Ames test. An auxotrophic histidine mutant of *S. typhimurium*, on the addition of a mutagen, will revert to wildtype, which leads to growth in the absence on histidine. Without the addition of a mutagen, the strain remains auxotrophic and no significant growth is observed.

Of the many new mutations that can occur on the addition of a mutagenic chemical to the mutant *S. typhimurium*, a small percentage will occur that compensate for the original mutation (Figure 1.5). As the Ames test incorporates a dense population of *S. typhimurium* cells, the small percentage of cells that undergo a compensatory mutation is significant and can be visualised as a greater number of colonies on the plate. These colonies are counted after incubation at 37°C for 2-3 days. The colony numbers are compared to negative control plates containing everything but the test chemical and positive control plates containing a strong mutagen such as benzo[a]pyrene (Plate 1.1).

The negative control plates will have a small number of revertant colonies growing on them due to spontaneous reversions at the genes encoding histidine synthesis. If the test plate contains at least a doubling of the number of revertants found on the negative

control plate at two consecutive doses (as with the positive control plate) the test chemical is considered mutagenic (Maron and Ames, 1983).



A B

PLATE 1.1. Colony revertants on negative (A) and positive (B) control plates in the Ames test. *S. typhimurium* TA98 on the addition of non-mutagenic DMSO carrier solvent (negative control) and 5 µg mL-1 mutagenic benzo[a]pyrene (positive control).

1.5.1.1 Bioactivation of Chemical Carcinogens in the Ames Test

In order to incorporate the phase I metabolic activation enzymes required for PAH mutagenicity into an *in vitro* experiment, a 9000 X g centrifugation supernatant of a rat liver extract called S-9 is used (Tauc *et al.,* 1984) along with the addition of nicotinamide adenine dinucleotide phosphate (NADP) as a co-enzyme. Aroclor-1254-induced rat enzymes were shown to be more effective than induced hamster S-9 (Raineri *et al.,* 1981) and were most readily available. Aroclor-1254-induced rat liver S-9 was described in De Flora *et al.* (1984) as being effective at activating carcinogenic PAHs in the Ames test. There are drawbacks to using this method however, as the enzymes are susceptible to changes in temperature and pH, which means that controls must be used to ensure the enzyme activity is optimised (Tauc *et al.,* 1984).

The activity of these enzymes has been shown to differ among mammalian species and even primate species, therefore creating problems in the extrapolation of results from animals S-9 to humans. For example, mouse cytochrome P450 1A1 metabolises Benzo[a]pyrene at a much greater rate that the human form (Shou *et al,* 1994). Human S-9 can be gained during autopsy therefore reducing extrapolation problems, but sources are limited and the enzyme activity may be reduced from this source (Josephy *et al,* 1997).

1.5.1.2 Key Factors and Modifications of the Ames Test

The Ames test relies on the fact that there are many available mutant alleles in the *Salmonella typhimurium his* operon, which are sensitive to mutagens. Many different sequence changes in the *his* operon will lead to a reversion to the wildtype (histidine producing). Such test strains were also modified to achieve greater cell permeability, as well as inactivating the DNA repair system so that damage was maximised. Each *S. typhimurium* tester strain used in the Ames test contains a different type of mutation in the *his* operon. For example, Strains such as TA1538 and TA98 have a frameshift mutation in the *hisD* gene (*hisD3052*) that encodes histidinol dehydrogenase (Maron and Ames, 1983).

The *S. typhimurium* strain found to be most sensitive to hydrocarbons was TA98 (Hermann *et al,* 1980). TA98 specifically includes mutation *rfa* (rough deep character) that causes partial loss of the lipopolysaccharide barrier that coats the surface of bacteria and increases permeability so that large molecules are able to enter. It also has the addition of a plasmid pKM101. The plasmid infers ampicillin resistance as a marker of the plasmid's stability (Maron and Ames, 1983) and encodes genes involved in error-prone DNA repair. There is a danger however, that by enhancing the mutagenicity, one may produce a false positive result. It is more dangerous to get a false negative result however, and for this reason enhancing mutagenicity remains an important tool (Josephy *et al,* 1997).

Blackburn *et al.* (1984, 1986) developed the Ames test into the form that is now known as the 'modified Ames test' and that has improved the sensitivity of the Ames test with respect to measuring the mutagenicity of the aromatic portion in oils. The modifications include an eight-fold increase in the concentration of S-9 and a two-fold increase in co-enzyme (NADP) to increase the probability of activation. The aromatic content of oil is also extracted using dimethylsulphoxide (DMSO), to concentrate the mutagens in the oil.

1.5.1.3 Alternative Mutagenicity Tests

Greater deviation from the Ames test is seen in the use of luminescent bacteria. One photobacterium bioassay commercially available is called the Mutatox™ test. *Vibrio fisheri* M169 will show an increase in luminescence on the addition of genotoxic chemical (Johnson, 1992; Bekaert *et al,* 1999). Commercially available tests such as Mutatox™ are expensive to both set up and use as they rely on a supplier to provide specialised components. Newer tests use genetically modified organisms, such as yeast (a eukaryote) with a green fluorescent jellyfish protein reporter plasmid added (Walmsley *et al,* 1997). However, all new tests must be validated using the Ames test as a standard. For these reasons, the Ames test was chosen for initial mutagenicity testing, especially with the modified method (Blackburn *et al.,* 1986). The Ames test can be set up in any hazard II microbiological laboratory, without great expense or specialised equipment. The test can also be performed on the bench and all components of the test can be prepared in-house.

1.5.2 Liquid-Liquid Extraction

Liquid-liquid extractions (LLE) are the main method of removing the aromatics from oil matrices and are either used solely or following a solid phase extraction (Paschke *et al.,* 1992; Moret and Conte, 2000, Wang *et al.,* 2000). The most commonly used LLE is the IP 346 method (BS2000 Part 346, 1996) which is the industry standard for the estimation of polyaromatic content in transformer oil, although it tends to overestimate

by measuring total aromatic content (Section 1.1.3.1). Oil aromatic content estimated by IP 346 is believed to correlate well with animal painting tests (Stang, 1993; 1999; 2000).

The general mechanism of LLE can be illustrated by the IP 346 % w/w extraction where an organic non-polar solvent (cyclohexane) is added to an aqueous polar solvent (DMSO and water) and the oil and its components are partitioned between the immiscible layers. As PAHs are polar they will prefer the DMSO layer, while the aliphatics prefer the organic layer. The mixture is shaken and left to separate, then more DMSO is added to ensure complete separation of the aromatics from the aliphatics. The aromatics can be collected in the DMSO layer or removed to a fresh organic layer by disrupting the DMSO layer with the addition of a salt solution. The method is outlined in Appendix B.

LLE has also been used with the Ames test to determine the mutagenicity of mineral oil (Blackburn *et al.,* 1986). Again, it was the aromatics that were removed, not just the PAHs, therefore mutagenicity measured by this extraction method may not automatically be attributed to PAHs.

1.5.3 Solid Phase Extraction

Solid phase extraction (SPE) is another sample preparation method. It has two main functions, to purify and concentrate an analyte. It was designed in the 1970's as an alternative to LLE as it has the benefits of better separation (due to no emulsion formation) and automation. SPE columns come in different formats, such as disks, for small quantities of sample (10-20 μL), cartridges for larger quantities (20-100 μL) which are most commonly used (shown in Plate 1.2) and finally barrels, which come in a large variety of sizes (Henry, 2000). The majority of formats are available in polypropylene with a matching frit. If platicizers are likely to interfere with the analysis, glass barrels with Teflon frits can be used.

When studying one compound in a sample, studies can be conducted to predict the extraction efficiency of an analyte according to its chemical properties. Such information can be used to determine the best sorbent to use. As oil is a complex mixture of compounds, however, prediction of such properties is unrealistic. Only a practical application of SPE will determine the best way to extract PAHs from oil.

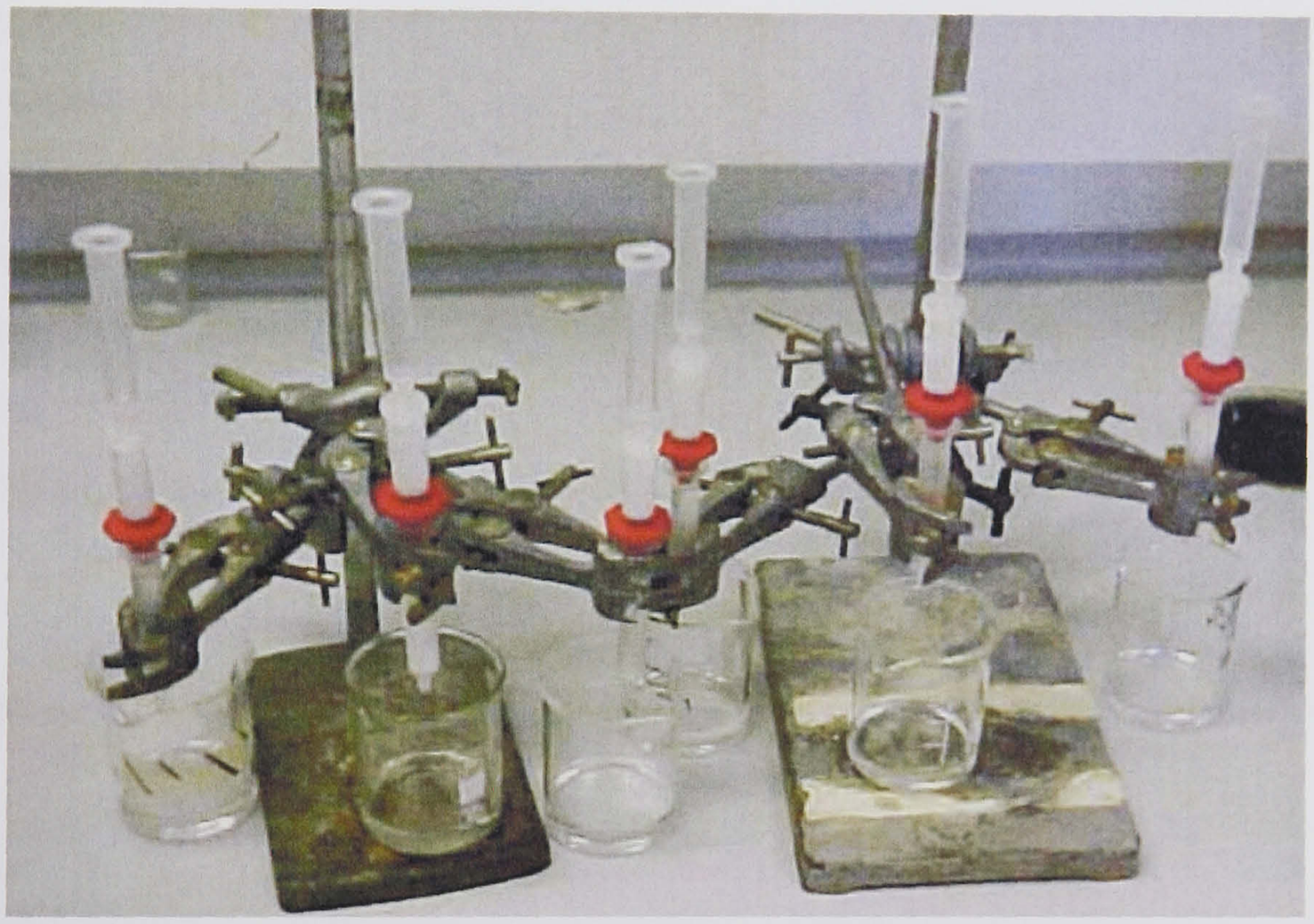


PLATE 1.2. SPE cartridges for solid phase extraction. The cartridges are shown in consecutive use with an adaptor (in red) to join the cartridges together. The top clear column is an unfilled filtration tube used as a solvent reservoir.

1.5.3.1 Types of SPE

Two types of SPE were used, normal and reverse phase. The most commonly used SPE for the extraction of components from oils is normal phase extraction. Normal phase extraction uses a polar sorbent to retain polar components within the column, therefore washing out non-polar alkane components. Washing is achieved by using a non-polar solvent. Most importantly, once the unwanted components have passed

through the column, the polar components can be removed using a suitable polar solvent (Moret and Conte, 2000). The ability of a solvent to remove the polar components from the column is measured as an eluotropic strength (E^{ϕ}). Choosing the correct solvent for elution will ensure effective extraction in the smallest possible volume, therefore ensuring a concentrated sample (Wells, 2000). Solvent eluotropic strength is taken from Thurman and Mills (1998) and shown in Table 1.3. An eluotropic strength of >0.6 was considered to be the most appropriate to elute moderately polar components from silica. An eluotropic strength of <0.38 was most appropriate for a washing solvent. However, as there were a number of polar components present in the oil, altering the solvent according to eluotropic strength allowed the separation of compounds with similar polarity. PAHs were found to only require a E^{ϕ} of ~0.43-0.45 for effective elution, as anything higher produced the elution of additional polar components including column bleed, which occurred with methanol (Section 4.2.2.1).

TABLE 1.3 Solvent eluotropic values. Adapted from Thurman and Mills (1998).

Solvent	*Eluotropic Strength (E^{ϕ})*	*Polarity*
Methanol	0.73	6.6
2-Propanol	0.63	4.3
Ethyl acetate	0.45	4.3
Acetone	0.43	5.4
Cyclohexane	0.03	0.0
n-Hexane	0.00	0.06

The most common normal phase sorbent used is silica (SiO_2). When SiO_2 is used to interact with the analyte, it does so with hydrogen bonding through a Si-OH bond. The silica gel can be modified by the addition of functional groups to produce sorbents with different properties including reverse phase extraction.

Reverse phase is literally the reverse of normal phase, using a non-polar sorbent, usually C18 (Silica with $(CH_2)_{17}CH_3$ groups attached). Bonding to the sorbent occurs through Van der Waals interactions. The advantages of the bonded silica sorbents are that they are stable in organic solvents, have good flow characteristics, and are easily wet with polar or non-polar solvents. Most importantly, a wide range of functional groups are available for various applications (Thurman and Mills, 1998).

1.5.3.2 Effects of Length and Width on SPE Columns

One important factor in effective SPE is the shape and size of the sorbent column. Plate theory is used in all chromatographic methods and relates the length and width of a column to its efficiency by describing the columns in terms of smaller sections called plates. As in all chromatographic methods, the sample slowly moves through the column with the solvent and will equilibrate with a small section of sorbent at a time. Plate theory was used for chromatography by Martin and Synge (1941) to predict the movement of an analyte. The primary finding was that the greater the number of plates in a column, the better the separation (Thurman and Mills, 1998).

As SPE columns are small in length compared to a column used in separation techniques such as gas chromatography, they will contain a small number of plates and therefore will not be efficient at separating analytes according to the distribution coefficient. However, they can still be used for isolation of analytes and the greater the number of theoretical plates present, the less dispersion that can occur and a lesser volume of solvent is required for elution. Increasing the amount of sorbent used however, will increase the volume of eluent required although when used in a thin column, this will also increase the plate number and therefore the efficiency of extraction (Thurman and Mills, 1998). The volume of sample added will also effect extraction efficiency. If too much sample is used, the column may become saturated and separation will not be complete. However, if too little is added, the analyte may interact more strongly with the unsaturated sorbent (if the analyte contains more than one binding site) and more solvent is required to remove it from the column. With

more analyte the interactions are shared between more molecules, and become easier to break.

An extension of plate theory, known as 'rate' theory is now used that acknowledges the contribution of elution rate on separation efficiency. Rate of elution will differ according to the analytes affinity for the column, the path through the stationary phase it takes (which may vary in length) and the extent of analyte diffusion (longitudinal diffusion) along a plate. Rate theory therefore illustrates the importance of constant flow rate as well as column length during chromatographic separations.

1.5.3.3 Sample Breakthrough

Another important factor in the development of SPE is the point at which the analyte begins to be lost (breakthrough) from the column. PAH breakthrough was easily determined as PAHs fluoresce. This allowed the monitoring of PAHs with an Ultra Violet (UV) lamp, and reduced the need for breakthrough experiments. However breakthrough during washing can be determined by continuous washing and analysis of the wash solvent after it is recovered from the column. Additionally, overloading of the column can also be established by running an extraction with different volumes of sample, and determining which produces the cleanest extract by gas chromatography (Simpson, 2000).

1.5.4 Gas Chromatography Mass Spectroscopy (GC-MS)

Quantitative analysis of oil is usually performed with techniques such as high pressure liquid chromatography (HPLC) and gas chromatography with mass spectroscopy (GC-MS), although earlier quantification was achieved only partially by the use of thin layer chromatography (TLC) with ultra-violet (UV) detection or fluorescence spectroscopy (Moret and Conte, 2000). HPLC in particular can determine the many isomers of PAHs and is used by the oil manufacturer Nynas for this purpose (Kroon, 1989). In the Nynas method the whole oil is dissolved in hexane and injected onto a

HPLC column of nitrile-substituted silica, which adsorbs aromatic rings with the compound with the greatest number of rings (most polar) adsorbing most strongly. The large PAHs stick so efficiently that backflushing is employed to remove them once the smaller PAHs are removed.

Recently, work has been performed with supercritical fluid extraction (SFE) as it removes the need for hazardous solvents and has a short analysis time. Much development of this technique is under way in the literature, and for PAH analysis it has been most effective after SPE (Marcé and Borrull, 2000). However, due to the limited availability of the HPLC and SFE equipment, the chosen analysis technique was GC-MS. GC-MS has proven to be particularly useful for PAH analysis as PAHs are thermally stable. The combination of the techniques (GC and MS) in particular, has the advantage of selective monitoring, which allows target compounds to be monitored with greater sensitivity even in complex mixtures. This may be of particular benefit for PAHs in oil, where the PAHs make up an extremely small fraction of the total components.

1.5.4.1 Gas Chromatogram (GC)

GC is a method for the separation of analytes in a gaseous mobile phase using a stationary phase. In the same way as SPE, the analytes interact with both phases, but are separated according to volatility. Volatile components spend little time on the solid phase and are therefore eluted quickly and said to have a short retention time. Only thermally stable analytes such as PAHs can be separated this way as the operating temperature ranges from 50-300°C, which would destroy many analytes and make detection impossible (McNair and Miller, 1998; Purdue University, 2002).

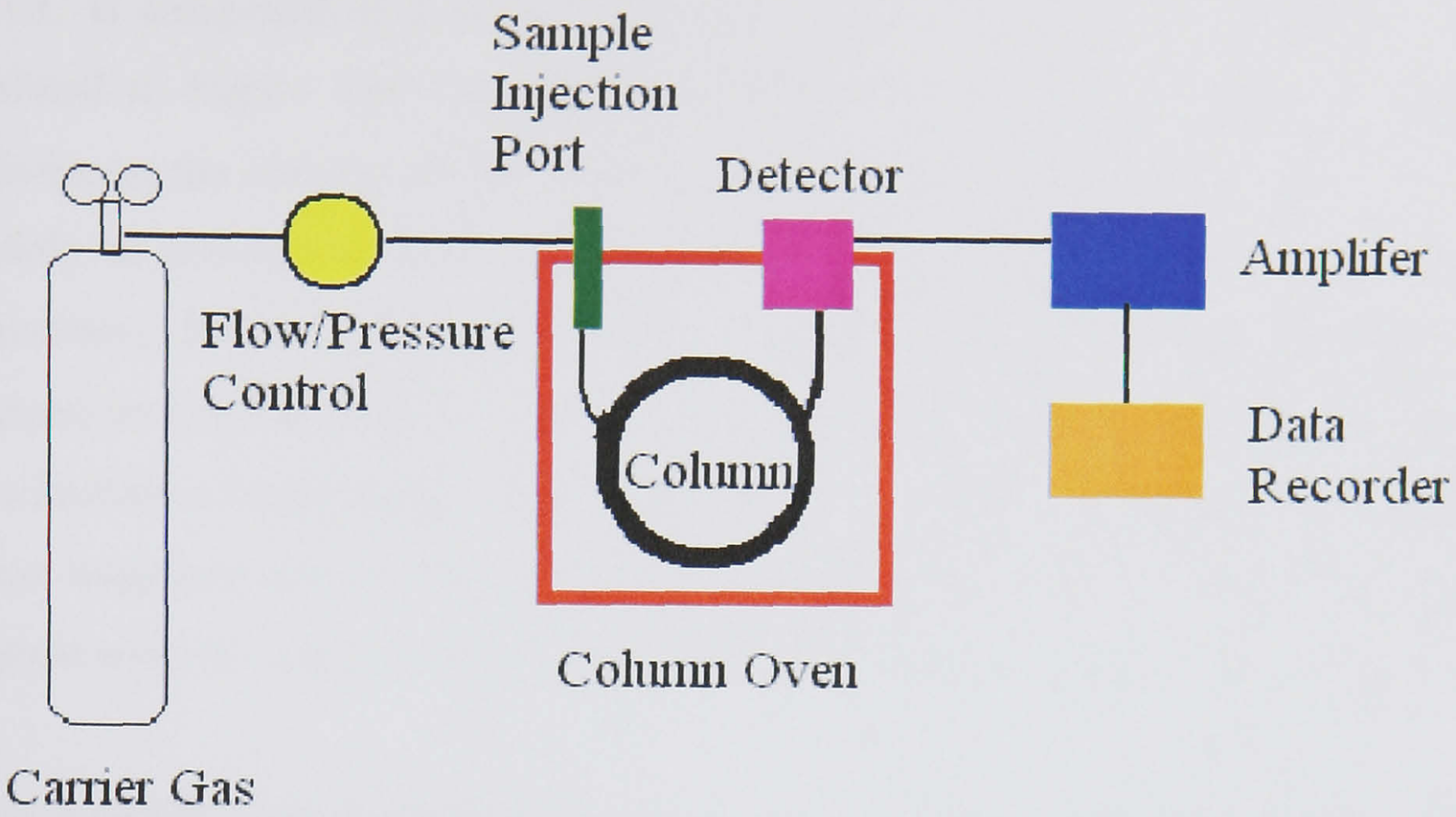


FIGURE 1.6. The principle components of a Gas Chromatogram (GC) (Purdue University, 2002).

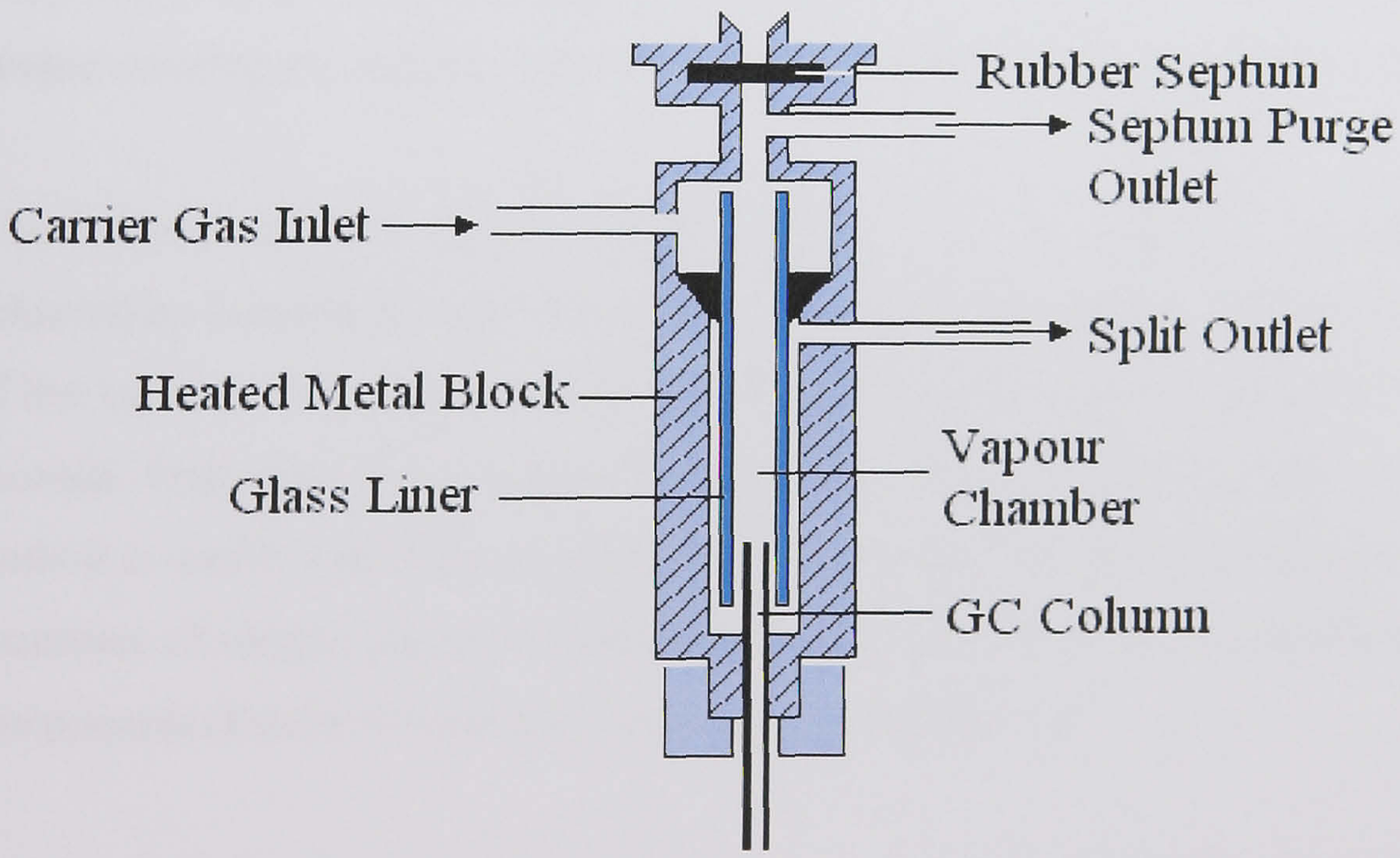


FIGURE 1.7. A GC slit/slitless injector (Polytechnic University, 2002).

A GC is composed of a number of parts crucial to the analysis of samples and are outlined in Figure 1.6. The first is the injection port, where sample is added. For efficiency, the sample should be added in low quantities (usually 0.1-2 μL) and as rapidly as possible to reduce peak broadening and improve resolution (Polytechnic University, 2002). The sample is made up in a volatile solvent such as methanol or acetone and injected with a syringe through a septum, which will seal once the needle is withdrawn. Unfortunately the septum can be the source of bleed as the needle cores some fragments into the column. The sample can also accumulate at the septum, so the septum must be changed regularly. A diagram of the injector is shown in Figure 1.7.

Two methods, split or splitless injection, can be used to inject the sample. The same injector is used in both cases and contains a glass liner with glass wool to increase the surface area allowing complete volatilisation. In split injection the sample is evaporated in the vaporisation chamber after injection and joins the carrier gas stream. Most of the sample is vented leaving 0.1-10% to enter the column. This method is used with simple mixtures, especially where the less volatile components are required for analysis, as highly volatile components tend to be vented. With such a small amount of sample entering the column, split injection is not reliable for quantitative analysis.

Alternatively, splitless injection allows up to 5 μL of sample to be added and is achieved by keeping the split valve closed and the column cooler than the boiling point of the solvent. The sample is injected slowly and allowed to evaporate for up to 60 seconds. Only then is the split valve opened to purge the injector port. The splitless method is useful with dirty samples as the glass liner can easily be replaced. As larger quantities of sample are used splitless injection is better for the quantification of trace components (Purdue University, 2002) such as PAHs in oil.

The second component of the GC is the column, which is the stationary phase. Columns can work at a range of 40-350°C. Capillary columns are hollow tubes with methylpolysiloxane (5% phenyl) coated onto the walls and are both flexible and inert. A smaller diameter and greater length is best due to a greater number of theoretical plates (Kitson *et al.*, 1996).

The third component is the carrier gas, which is the mobile phase. The carrier gas must be inert and preferably of no risk to health so helium, hydrogen or nitrogen are most often used (helium in this case). The carrier gas mixes with the sample in the injection chamber and carries it through the column. The flow rate must be kept constant irrespective of the temperature for effective separation and repeatability (Purdue University, 2002).

The oven and other temperature control devices make up the forth important component. Without such heating devices the sample would not volatilise. Without correct heating at every part of the system the sample would condense, leading to poor separation and contamination of the system. The injector temperature is usually around 50°C higher than the boiling point of the least volatile component of the sample (Polytechnic University, 2002).

Controlling the column oven temperature allows effective separation of sample components. The column is usually maintained for a short time at a low temperature at the start of an analysis to allow the highly volatile components to elute effectively. If the temperature were too high at this point, all the volatiles would elute together giving poor resolution. The temperature is then ramped to up to 40°C min^{-1} to allow volatilisation of the less volatile components. The higher the temperature, the faster the elution, but by carefully ramping the temperature the separation and resolution of a mixture is not compromised (Purdue University, 2002).

In GC, the column would enter a detector and recorder to collect the data. However, in GC-MS the capillary column exit is inserted into the ion source of the mass spectrometer.

1.5.4.2 Mass Spectrometer (MS)

When the sample leaves the GC column it enters the ion source of the MS, which is under vacuum. The ion source used here converts the sample into gaseous ions by bombardment of the analyte with electrons (electron impact, EI). The electrons have a kinetic energy of ~70 eV which allows it to knock electrons out of the analyte molecules. This leads to the formation of cations, which are accelerated towards a mass analyser by a potential (a few volts), which separates the ions according to their mass/charge (m/z) ratios (West Virginia University, 2002).

The mass analyser used in this work was a quadrupole, a group of four electromagnets, and is shown in Figure 1.8. The electrically charged poles create a magnetic field that aligns the ions. Masses can be selected by sweeping a radiofrequency signal. With each frequency a different m/z ratio can escape the quadrupole and hit the detector. Sweeping across a range, from high to low, releases the range of m/z ratios individually, to produce a spectrum, by plotting m/z ratio against intensity (abundance). When GC is combined with MS, a measure of time vs. intensity vs. m/z ratio is achieved which allows each separated GC peak to be identified by its specific MS spectrum. A total ion chromatogram (TIC) is produced when all the raw signals are plotted against time. Alternatively, ions of interest can be selected and plotted against time in a selective ion chromatogram (SIC) (Niessen, 2001).

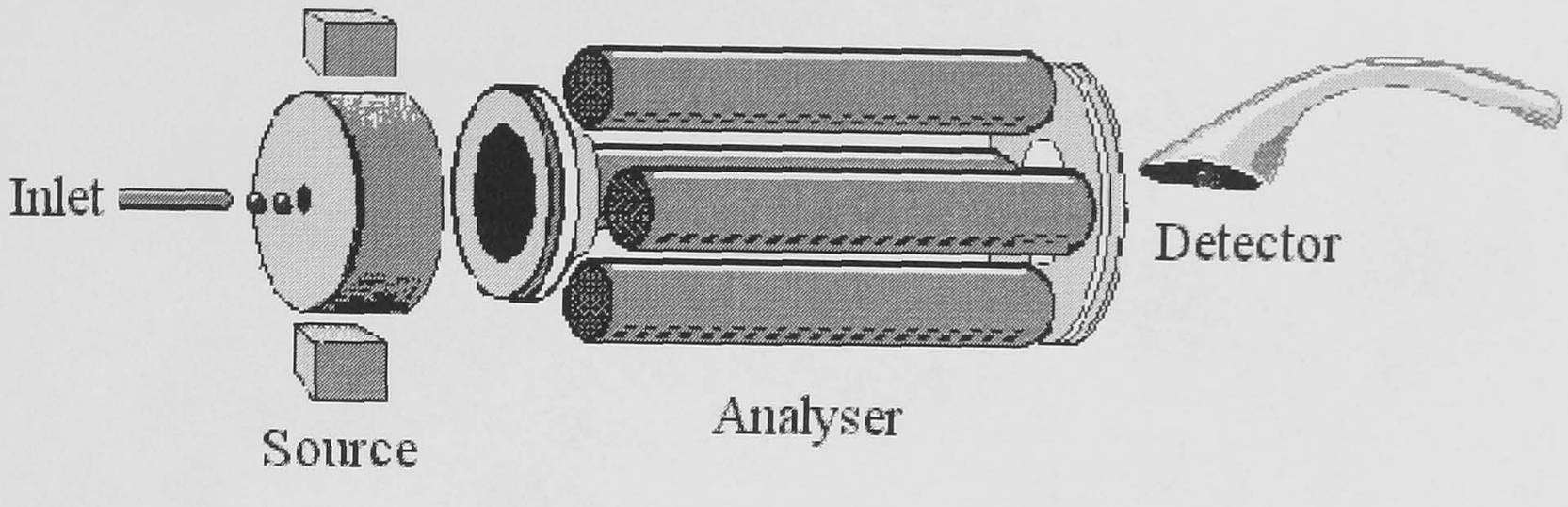


FIGURE 1.8. The principle components of a Mass Spectrometer (MS) (Polytechnic University, 2002). The inlet is where the GC column enters the ion source and the analyser is a quadrupole.

1.5.5 Immunoassay

Immunoassays use antibodies in the detection or isolation of an analyte. As antibodies are specific to antigens, the analyte is injected into an animal, such as a sheep or rabbit to elicit a response from the animal's immune system. The antibodies produced are isolated and used, in this case with a labelled analogue as a measure of the PAH present. Most work conducted using antibodies to detect PAHs has been performed in water and soil samples using commercially available kits (Barceló *et al.,* 1998; *Chuang et al.,* 1998). Commercial kits are easier to use, although costly, as setting up and optimising an immunoassay can take years of research.

A commercial kit was used as an additional PAH detection method. Although there were concerns about how it would perform in a complex oil matrix it had been successfully used during previous studies at Cranfield University by Kim *et al.* (2001) with great dilution of the oil matrix. The mechanism involved in the immunoassay method is summarized in Figure 1.9. More detail on the test is outlined in Section 2.1.6 and 2.2.13.

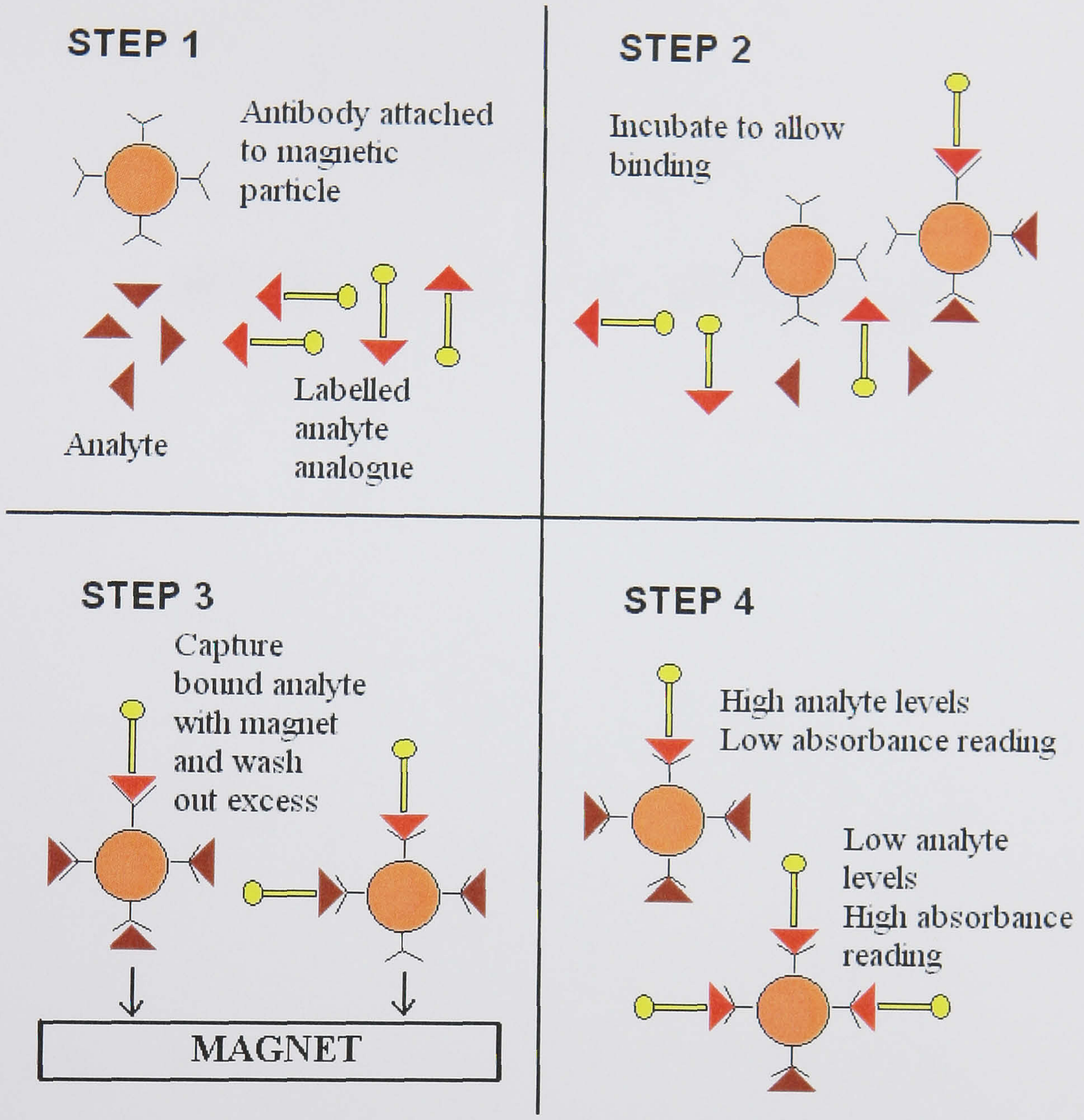


FIGURE 1.9. Outline of the competitive PAH immunoassay kit. (1) Mixing of the sample with the antibody/magnetic particle and colour labelled analyte analogue. (2) Incubation, the analyte and analogue compete for binding sites of the antibody. (3) Washing of unbound analyte and analogue while the antibody/magnetic particle is held with a magnet. (4) Coloured label is read by absorbance at 450 nm. Absorbance is inversely proportional to analyte.

CHAPTER 2.0

MATERIALS AND METHODS

2.1 MATERIALS

The materials and preparation steps for the techniques used in this work are set out below. The method for each technique is outlined in detail in Section 2.2.

2.1.1 Oils Samples

All oils were supplied by the National Grid Company Plc. Supplied were:

- Twelve oils, with varying aromatic content from three different manufacturers (details in Table 2.1)
- Nytro-10GBN (N10GBN), the most commonly used transformer oil by the National Grid Company Plc
- A sample of Castrol BS148 which was artificially aged for three weeks
- The highly refined 'white' oil serving as a PAH-free control. However, it differed from transformer oil in its composition due to the extensive refining process.

The aged oil was provided to determine how PAH composition changes with use. Changes in PAH composition may affect oil oxidation stability and mutagenicity. The original oil will be referred to as 'ALT 0'. ALT 0 oil was artificially aged using a method recognised by the electrical power transmission industry (BS EN 61125, 1993) for the National Grid Company Plc at the Glasgow Caledonian University. Aluminium electrodes were immersed in the oil and the voltage increased until electrical discharges were observed. This voltage was applied for 1 week (ALT 1) 2 weeks (ALT 2) and 3 weeks (ALT 3) producing a total of 3 aged oils for comparison with ALT 0.

TABLE 2.1. Details of the transformer oil samples 1-12 and Nytro-10GBN. All oils were provided by the National Grid Company Plc

Oil Identification Number	*IP 346 (% v/v)*	*Manufacturer*
1	1.8	Nynas
2	2.1	Carless
3	3.3	Nynas
4	<1.0	Carless
5	2.6	Carless
6	1.8	Carless
7	1.0	Nynas
8	8.9	Carless
9	2.2	Carless
10	4.5	Carless
11	<1.0	Nynas
12	3.7	Castrol
Nytro-10GBN	1.75-2.64*	Nynas

** IP 346 value measured in house*

Oil 8 contained the greatest amount of PAH according to the IP 346 % w/w data and was therefore chosen for initial testing. In addition oil 4, which had a particularly low PAH content according to IP 346 % w/w data, was tested along with Nytro-10GBN which was tested due to its abundant use. White oil was also used in experiments to give a background 'blank' reading.

2.1.2 The Ames Test

All components of the Ames test were purchased from Sigma-Aldrich (Poole, UK) unless otherwise stated. The procedure was slightly modified from that described in Maron and Ames (1983) to simplify preparation and was performed in a Hazard II safety cabinet. Dimethyl sulphoxide (DMSO, 99+% ACS reagent) was purchased from Fisher Scientific Ltd (Manchester, UK).

2.1.2.1 Inoculation of Bacteria from Original Freeze Dried Sample

Nutrient broth was prepared using Nutrient Broth No. 2 BP pH 7.3 (2.5g) purchased from Lab M (owned by IDG Ltd, Bury, UK) in deionised water (100 mL). After autoclave sterilisation it was inoculated with a freeze dried *Salmonella typhimurium* TA98 strain (Public Health Laboratory Services Culture Collection, London, UK). The culture was shaken at 30°C in a 100 mL conical flask and grown up for 16 hours. A small quantity of this culture was propagated on a nutrient agar plate until the liquid culture was deemed satisfactory. If the liquid culture was contaminated, a colony from the agar plate could be used to produce a new culture, by inoculation into fresh sterilised broth.

Frozen 1 mL vials of culture were prepared for future use by adding 90 μL of DMSO per 1 mL of culture to the original nutrient broth culture. Each aliquot was stored in a microtube (BDH, Poole, UK) and frozen at –80°C. To make a fresh culture from a frozen vial, fresh nutrient broth (100 mL) was inoculated with the thawed culture and shaken for 16 hours at 30°C in a sterilised conical flask. The fresh culture was kept in the fridge for up to 5 days.

Routine checks to ensure the desired phenotypes (discussed in Section 1.5.1.2) are intact (ampicillin resistance, histidine requirement) were performed on nutrient agar plates, once every 3 months or whenever the bacteria demonstrated an unexpected characteristic, as described in Maron and Ames (1983) (Appendix A).

2.1.2.2 Preparation of Minimal Glucose Vogel Bonner (VB) Plates

The Vogel Bonner (VB) solution (a minimal glucose, high salt composition) was made up as outlined in Appendix A (Maron and Ames, 1983). The plates were prepared in 100 mm x 15 mm petri dishes (BDH) and contained 30 mL of minimal glucose VB agar medium. The medium was prepared by adding 7.5 g agar to a 1 L Duran bottle (BDH), with 490 mL de-ionised water and the 10 mL VB solution. This was

autoclaved for twenty minutes at 121°C and within 10 minutes of cooling, 10 g glucose was added directly to the solution and mixed slowly by swirling the bottle by hand to minimise foaming. The plates were stored at 4°C for up to a month before use.

2.1.2.3 Preparation of the S-9 Mix

Phosphate buffer solution (pH 7.4) was prepared as outlined in Appendix A (Maron and Ames, 1983). The amounts of each component per 10 mL of the S-9 mix (10% v/v) were as follows:

5.0 mL Phosphate buffer (pH 7.4; 0.2 M)
4 mL De-ionised Water
1 mL Rat liver S9 (Aroclor-1254-induced)
14.1 mg Glucose-6-phosphate
31 mg Nicotinamide adenine dinucleotide phosphate (NADP)

Rat liver S-9 homogenate was purchased from ICN (Basingstoke, UK). The phosphate buffer and distilled water for the S-9 mix were sterilised by autoclaving for 20 minutes at 121°C. NADP and glucose-6-phophate were added to the buffer water mixture almost immediately after autoclaving. The S-9 was added once the solution had cooled to 40°C.

For the "Modified Ames Test" described by Blackburn *et al.* (1984), twice as much NADP (62 mg) and glucose-6-phosphate (28.2 mg) were used and 8 mL of S-9 was added, replacing both water and buffer. This ensured optimum activation of the PAHs in the oil sample by the enzymes. The volume was made up to 10 mL by the addition of 2 mL of buffer. The S-9 mix was freshly prepared each day and kept on ice before use.

A S-9 free solution was also prepared to determine the extent of sample mutagenicity without enzyme activation (direct mutagenicity). This buffer was prepared in the same way as the 10% v/v S-9 mix. with de-ionised water replacing the S-9.

2.1.2.4 Preparation of Top Agar

The 0.5mM histidine and biotin solution used in the top agar was prepared as described by Maron and Ames (1983) and outlined in Appendix A.

The top agar was prepared as follows:

0.6 g Agar

0.5 g Sodium chloride

90 mL Distilled water

10 mL Histidine Biotin solution (0.5mM)

The top agar was autoclaved for 20 minutes at 121°C prior to use and stored in a water bath at 45°C until required.

2.1.3 Liquid-Liquid Extractions

All solvents (99+% ACS reagent) were purchased from Fisher Scientific Ltd and all separating funnels were from BDH. Evaporation was achieved using a rotary evaporator (BUCHI Rotovapor R-110).

2.1.4 Solid Phase Extraction

Spiking experiments were performed using a standard containing 10 μg mL^{-1} of each of the EPA 16 priority PAHs in cyclohexane supplied by Qmx Laboratories (Essex, UK). The Camag Ultra Violet (UV) lamp used to track the PAH fluorescence (at 366 nm) was purchased from BDH.

Silica gel (60Å) and Aluminium oxide (neutral alumina) was purchased from Sigma-Aldrich (Poole, UK). The 900 mg Cyanopropyl and 900 mg C18 cartridges were purchased from Machery-Nagel (Oxon, UK). The 1 g PAH HC Isolute SPE columns were purchased from Jones Chromatography (Hengoed, UK). Polypropylene filtration tubes (1 cm diameter, 3 mL length) with matching polyethylene frits were supplied by Supelco (Bellefonte, USA).

The 20 mm x 200 mm glass chromatography columns briefly used to assess the effects of column thickness (Section 4.2.2.3) were purchased from BDH. The preferred 10 mm x 460 mm glass Ace chromatography column used in Section 4.3.1 with fritted disc was purchased from Sigma-Aldrich as was the larger 50 mm x 600 mm glass chromatography column used to extract 20 g of oil in Section 3.6.

Quickfit glass adaptors were used to attach a syringe or a compressed air tap to the columns, to increase pressure and therefore increase solvent elution rate. The size of the adaptor varied depending on the column used but all quickfit adaptors were purchased from BDH except for the large scale extraction column, the adaptor for which was purchased from Sigma-Aldrich. The adaptors used with the small scale polyethylene columns were PTFE column adaptors purchased from Jones Chromatography.

2.1.5 Toxicity Measurements

Sterile, flat bottomed 96 well microtitre plate and microwell sealing tape were purchased from BDH. A computer controlled Dynex Technologies Ltd MRX plate reader (Billingshurst, UK) was used to read absorbance at 600 nm.

2.1.6 Immunoassay

Two RaPID assay PAH test kits were purchased from Strategic Diagnostics Inc (Newark, DE USA). One was for total PAH measurement and the other favoured the detection of carcinogenic PAHs. All reagents were supplied with the kits and are listed in Appendix A.

Polystyrene test tubes were also included, and all reagents contained stabilisers and preservatives to increase the shelf life of the kits. The oils were diluted in methanol (Sigma-Aldrich) before testing.

Paramagnetic beads were separated from the sample solutions using a magnetic separation rack (Strategic Diagnostics Inc). Colour development was measured using a plate reader (Dynex Technologies Ltd).

2.2 METHODS

In this section, the methods used in this work are outlined in detail. The materials used in this section have been discussed in Section 2.1.

2.2.1 Ames Test Sample Preparation

The samples were usually tested over 5 doses (10 fold dilution in each case) to test for mutagenicity over a wide range (Organisation for Economic Co-operation and Development (OECD), 1983). A dose response, particularly with a doubling of the reversion number observed on the negative control plates (at 2 consecutive dilutions) indicated mutagenicity (Jackson and Pertel, 1986). A doubling observed at only one dilution was justified by additional testing over a smaller dose range. This standard of mutagenicity was difficult to adhere to for the liquid-liquid extracts of oil, as the highest dose available for testing, was the only dose that produced a mutagenic

response. If a higher dose could not be achieved for mutagenicity testing, the results were validated by comparison with the mutagenicity data of alternative extracts of the same oil. In addition, testing over 5 doses was occasionally limited to a smaller range when the S-9 concentration was increased to 80% v/v, due to limits in S-9 availability.

Indirect mutagenicity (activation required) was measured with the S-9 solution (Section 2.1.2.4), while direct mutagenicity (no activation required) was measured using a S-9 free solution (OECD guidelines, 1983). Each dose of samples was tested for direct and indirect mutagenicity in triplicate (6 plates in total).

Controls were included to ensure that the test bacteria were growing as expected. Positive control plates included a known mutagen (50 μg mL^{-1} benzo[a]pyrene or 20 μg mL^{-1} 2-amino anthracene) to indicate a mutagenic response. For a negative control, plates were run with only DMSO to indicate the background spontaneous revertants produced irrespective of sample. Both positive and negative plates were run with and without S-9 as indicated in Table 2.2. If these plates did not produce the excepted number of revertants, the accompanying test samples would be repeated.

TABLE 2.2. The contents of the negative (non-mutagen) and positive (mutagen with 10% v/v S-9) control plates incorporated into top agar in the Ames test. The presence of each component is indicated with a cross.

Plate Number	*Type of Control*	*S-9*	*DMSO*	*B[a]p*	*2-aa*	*Revertant Number*
1 to 3	Negative		X			20-40
4 to 6	Negative	X	X			20-40
7 to 9	Positive	X		X		200-300
10 to 12	Positive	X			X	400-600

Where reversion numbers were to be directly compared e.g. oil 8 with Nytro-10GBN, white oil and oil 4, all Ames tests were performed using the same batch of *S. typhimurium* culture to minimise the problem of inter-batch variability.

2.2.2 The Ames Test Procedure

The Ames test was performed in a Hazard II safety cabinet. Up to 70 sterilised universals were set out in rows of six at one time. The caps of the universals were unscrewed but kept on to prevent contamination. All samples were run with and without S-9 in triplicate. Each set of experiments was then repeated on another day, to check inter-batch variability.

To each universal the following was added:
0.1 mL a 16 hour nutrient broth culture of S. typhimurium
0.1 mL sample to be tested, in DMSO
0.5 mL S-9 mix, or buffer

The contents of each universal was gently shaken by hand and left for 20 minutes to allow activation to begin. A 2 mL volume of molten top agar, kept at 45°C in a water bath was added to the first three universals and shaken gently by hand.

The contents of the first three universals were individually poured from the universals onto VB minimal glucose plates. The lids were replaced and distribution of the top agar onto the surface of the plate was made uniform by tilting and rotating and placing the plate on a flat surface until solid. The addition of top agar and plating was then repeated for all plates, three at a time.

When all the plates were poured, they were inverted and stored in sterile plastic bags. They were then incubated at 37°C within 1 hour of plating. Growth occurred over 48 hours. After 48 hours the colonies (revertants) in both control and test plates were counted and the presence of the light background lawn of growth (due to the trace of histidine present in the media) confirmed.

The dose is reported as milligrams of oil extracted rather than milligrams of oil extract and is plotted against the mean number of revertants per plate, as is the convention in the literature (Herman *et al.*, 1981; Granella *et al.*, 1991, 1995; Brooks *et al.*, 1995).

This allowed a simpler comparison of whole oil mutagenicity results with oil extract results.

2.2.3 Liquid-Liquid Extractions

2.2.3.1 IP 346

The gravimetric IP 346 % w/w procedure was executed as described in method BS2000 Part 346 (1996). The method is outlined in Appendix B. Oil 8, 4 and Nytro-10GBN were extracted, 2.5 g of oil was used per extraction. Cyclohexane, dimethyl sulphoxide and a NaCl solution (4% v/v) were used to extract the aromatic fraction. For safety reasons, only 2-3 g of oil could be extracted at one time due to the large quantities of solvent required. The method was repeated four times for each oil to determine repeatability. The oil was weighed before and after extraction to establish the percentage aromatic fraction remaining (% w/w). The oil extracts were evaporated to dryness with a rotary evaporator. The residue from 2 extractions per oil was combined for Ames testing in 1 mL of DMSO.

2.2.3.2 The Grimmer Method

The Grimmer method is a liquid-liquid extraction modified from the method described by Grimmer *et al.* (1981). A sample of 5 g of oil, 18 mL DMSO, 2 mL cyclohexane and 2 mL de-ionised water was added to a 100 mL separating funnel. This was shaken vigorously for 1 minute and left to stand until the two immiscible layers had completely separated. The time taken to separate depended on the purity of the oil. The lower aqueous layer was removed and added to a second 250 mL capacity separating funnel containing 50 mL cyclohexane and 25 mL deionised water. The funnel was shaken for 1 minute and the layers left to separate. The lower aqueous layer was discarded while the upper organic layer was evaporated to 10 mL by a rotary evaporator, then further evaporated to 1 mL at room temperature in a fume cupboard. The extract was reconstituted in 1 mL of DMSO for subsequent Ames testing.

2.2.3.3 The Blackburn Extraction

The extraction method was performed as described by Blackburn *et al.* (1986) but scaled up to extract 6 mL (~5 g) of oil instead of 2 mL. The extraction mixes 6 mL of oil with 9 mL of cyclohexane and 30 mL of DMSO. The mixture was shaken vigorously by hand for 20 seconds every five minutes, for at total of 30 minutes and the DMSO layer was recovered for mutagenicity testing.

2.2.4 Solid Phase Extraction

The initial preparative methods used to extract PAHs using solid phase extraction (SPE) was based on the approach by Wang *et al.* (2000). However, since hexane and dichloromethane were considered too hazardous, cyclohexane and ethyl acetate replaced them respectively. Silica sorbent was used as it was polar and would interact with PAHs allowing them to be collected after the removal of unwanted, less polar oil components. Three extraction approaches were evaluated. (1) a 10 mm x 46 cm column containing 15 g of sorbent used to fractionate liquid-liquid extracts (Section 3.7) or to test sorbent extraction efficiency (Section 4.3.1), (2) a 50 mm x 600 mm glass column with 182 g of sorbent used to extract large quantities of oil and therefore allow a greater range of doses to be tested (section 3.6) and (3) small scale cartridges or columns containing 09-1.2 g of sorbent for extraction development (Section 4.5).

2.2.4.1 SPE with 15 g Columns

A sorbent weight of 15 g of silica (SiO_2) or alumina (Al_2O_3) was added to the 10 mm x 46 cm glass column and gently rotated in the palms of the hands to ensure even column packing. A weight of 1 g of oil or the extract of 1 g of oil (from a previous extraction) was added to the top of the silica column after conditioning with 20 mL cyclohexane. A 60 mL volume of cyclohexane was used to displace the extract down

the column and removed many unwanted components. Using an UV lamp at 366 nm, the fluorescence of the PAHs was tracked down the column to ensure that significant quantities of PAHs were not eluted during washing. If PAH elution was suspected the cyclohexane volume was reduced accordingly. All cyclohexane washes were discarded after use. Smooth elution was enhanced using a 20 mL syringe attached by plastic tubing to a quickfit adaptor to give a flow rate of approximately 2-3 mL min^{-1}.

Typically 2 bands of fluorescence formed on the silica, both fluorescing at 366 nm. These bands were eluted separately by using 2 x 30 mL ethyl acetate or acetone. The UV lamp was once again used to ensure complete band elution. The syringe method was used to ensure a flow rate of approximately 3-4 mL min^{-1}. The eluted fractions were evaporated to 10 mL using a rotary evaporator then evaporated to dryness in a fume hood at room temperature. The sample was then reconstituted in 1 mL DMSO for subsequent Ames testing.

2.2.4.2 SPE with 182 g Columns

A 50 mm x 600 mm glass column was used to extract 20 g of oil. Approximately 182 g of dry silica was poured into the column and gently rotated to ensure even column packing. Further removal of voids was achieved by tapping the column with a piece of plastic tubing. The column was conditioned with 50 mL cyclohexane and the 20 g oil sample was added to the top of the silica. A 800 mL volume of cyclohexane was used to wash the non-polar components of the oil from the column. The flow rate was maintained at 3-4 mL min^{-1} by the addition of the appropriate quickfit adaptor to the top of the column, attached by plastic tubing to a compressed air tap. All cyclohexane was discarded after use. Two bands of aromatic components, fluorescing strongly at 366 nm were observed when an UV lamp was used. A 2 x 60 mL volume of ethyl acetate eluted the two aromatic bands from the column. The flow rate was approximately 4-5 mL min^{-1}. The solvent was evaporated to 10 mL using a rotary evaporator and then to dryness in a fume hood at room temperature. The sample was then reconstituted in 1 mL DMSO for subsequent Ames testing.

2.2.4.3 SPE with 1 g Columns

Filtration tubes (1 cm diameter, 3 mL length) pre-prepared with base frits, were filled with silica (1.2g; equivalent to 2.5 mL) and conditioned with 8 mL cyclohexane. A 100 μL volume of oil was used unless otherwise stated. Cyclohexane was used to elute non-polar interference (flow rate 2-3 mL min^{-1}) followed by 6 mL acetone to elute the aromatic fractions (flow rate 4-5 mL min^{-1}). An empty filtration tube was attached to the top of the column, to act as a reservoir for solvent, and flow rate was controlled by the addition of a 10 mL syringe linked to the top of the solvent reservoir by an adaptor. The acetone eluent was evaporated to a final volume of 1 mL at room temperature in a fume cupboard prior to GC-MS analysis. A rotary evaporator was not used due to minimal solvent.

2.2.5 Investigation of Oil Interference with Liquid Media Ames Test

A volume of 0.1 mL *S. typhimurium* culture, 0.1 mL DMSO and 0.5 mL S-9 mix (10% v/v) was placed in sterile universals (numbered 1-6). Instead of adding a top agar media that was plated onto a solid VB plate, a 3 mL VB/top agar mix in liquid form was added.

The VB solution was comprised of the following:
2 mL VB salt solution used in the Ames test (Appendix A)
4 g Glucose
10 mL histidine/biotin solution (0.5 mM) (Appendix A),
De-ionised water to make a volume of 100 mL

A 100 mL of VB solution (without glucose) was autoclaved in a 250 mL Duran bottle at 120°C for 20 minutes and cooled to <40°C. Glucose was added ~10 minutes after autoclaving by swirling the Duran by hand. To universals 1 and 2, 50 μL Nytro-10GBN oil was added and the universals were incubated for 24 hr at 37°C.

After incubation, 0.5 mL of the mixture in universal 1-6 was added to fresh VB liquid media (4.5 mL) and aliquots (200 μL) were placed in 2 rows of wells in a sterile microtitre plate (16 repeats). Oil Nytro-10GBN was added to universals 3 and 4 (50 μL) after transfer to the microtitre plate. The microtitre plate were sealed with microwell sealing tape and placed in the incubator at 37° C for 24 hours.

Finally, Nytro-10GBN oil was added to the wells that originated from universals 1 and 2 (currently oil free) and 5 and 6 immediately prior to reading the absorbance at 600 nm on the plate reader. This final addition of oil ensured that any oil affects on absorbance readings were taken into account.

2.2.6 GC-MS Analysis

2.2.6.1 Perkin Elmer System

SPE extracts were analysed using a Perkin-Elmer Turbomass GC mass spectrometer with NIST library (Buckinghamshire, UK) unless otherwise stated. The column used was a Perkin-Elmer Elite series 5MS (30 m × 0.25 mm, 25 μm film thickness) with splitless injection. The injection volume was 1 μL and the injector and GC-MS interface temperatures were both 300°C. The oven temperature was held at 55°C for 2 minutes then ramped (30°C min^{-1}) to 180°C. Finally the temperature was ramped to 310°C at a rate of 5°C min^{-1} and held for 5 minutes. The mass spectroscopy was performed in electron ionisation (EI) mode (70eV) with source temperature at 230°C and scanned from 30-350 atomic mass units for a scan time of 0.2 sec^{-1} in total ion chromatogram (TIC) mode.

Selective ion chromatograms (SIC) were collected for quantification of the EPA 16 priority PAHs with a dwell time of 0.1 seconds. Mass and retention times programmed for SIC are shown in Table 2.3. All GC parameters for SIC were the same as those for TIC. Initial quantitative data was determined with the Turbomass quantitative software, which created a calibration curve from the standards run and automatically

determines the amount of each PAH present in the sample. Linear calibration curves were obtained for each of the 16 EPA priority PAHs across a 5-point calibration range 0-2 $\mu g\ mL^{-1}$. The software did not calculate the error related to linear regression. Therefore, once the final solid phase extraction was developed, quantitative data was obtained by manual integration of peaks so that the calibration curve and linear regression analysis could be determined.

2.2.6.2 Hewlett Packard System

A Hewlett Packard GC5890 series II with a HP MSD 5971A detector was also used in this work. The column was a HP5-MS (30 m × 0.25 mm, 25 μm film thickness) with splitless injection. The GC and MS programmes for TIC and SIC were as outlined for the PE Turbomass (Section 2.2.6.1). All quantification on the HP GC-MS was performed by manually integrating peaks, drawing a 5-point calibration curve 0.05-1 μgmL^{-1} and calculating the concentration of unknown samples using spreadsheets created in Microsoft Excel.

TABLE 2.3. GC-MS retention windows for the selective ion chromatogram (SIC) of the EPA 16 priority PAHs

Name of PAH	*Mass*	*Retention Window*
Naphthalene	128	4.61-7.84
Acenaphthylene	152	7.38-7.57
Acenaphthene	154	7.62-7.76
Fluorene	166	7.83-9.19
Phenanthrene	178	10.04-10.72
Anthracene	178	10.04-10.72
Fluoranthene	202	13.58-15.18
Pyrene	202	13.58-15.18
Benzo(a)anthracene	228	18.38-21.19
Chrysene	228	18.38-21.19
Benzo(b)fluoranthene	252	22.10-27.91
Benzo(k)fluoranthene	252	22.10-27.91
Benzo(a)pyrene	252	22.10-27.91
Indeno(1,2,3-c,d)pyrene	276	25.05-31.95
Dibenz(a,h)anthracene	278	25.05-31.95
Benzo(g,h,i)perylene	276	25.05-31.95

2.2.7 The Isolute PAH HC Protocol

The Isolute PAH PC protocol was designed for the removal of PAHs from soil samples (Jones Chromatography). The method was therefore modified to accommodate oil matrix effects.

2.2.7.1 Manufacturer's Method

A 100 μL volume of oil was added to the column after conditioning with 8 mL hexane and allowed to pass through by gravity flow. A 3 mL volume of pentane was added to

remove unwanted components using gravity flow followed by 6 mL hexane (containing 3.4% v/v isopropanol) to elute the PAHs from the column.

2.2.7.2 Modified Method

The oil sample was added to the column in 0.5 mL cyclohexane after column conditioning with 10 mL cyclohexane. A 2 mL volume of pentane was then added to wash out further unwanted components under a slight positive pressure (flow rate 1 mL min^{-1}) and the eluent discarded. PAHs were eluted with 6 mL acetone under pressure (flow rate 2 mL min^{-1}) and concentrated to 1 mL by evaporation at room temperature in a fume cupboard.

2.2.8 The Mineral Insulating Oil Fingerprinting Technique

The method used is described by Wilson and Pahlavanpour (2000) but modifications were made after discussions with the authors, to improve repeatability. For this reason the liquid-liquid extraction procedure is outlined in Appendix B.

2.2.9 C18/Silica/Isolute PAH HC Extraction

The extraction process is outlined in Figure 2.1. Silica (SiO_2) columns were prepared in-house by placing the accompanying frit in the base of the 3 mL filtration tube and adding dry silica gel (1.3 g, equivalent to 2.5 mL). The tip of the column was placed on a vortex at its highest speed for 10 seconds to reduce void formation.

Commercial silica columns of this sorbent weight usually come in a 6 mL column, 2 cm in diameter. In order to increase the plate number while still using only 1 g of sorbent, a narrower (1 cm diameter) 3 mL filtration column was used. This also prevented drying out of the column, as it reduced the sorbent surface area exposed to the air at the top of the column.

The column was conditioned with 4mL cyclohexane (4-5 mL min^{-1}) and then placed below the C18 cartridge. The C18/SiO_2 columns were then conditioned with 8 mL cyclohexane, which was discarded after use. A 100 µL aliquot of transformer oil was loaded onto the C18 column and allowed to interact with the sorbent for 1 minute. The C18 cartridge was covered to prevent the sorbent drying out. Samples were eluted onto the SiO_2 column with 3 mL cyclohexane (1-2 mL min^{-1}).

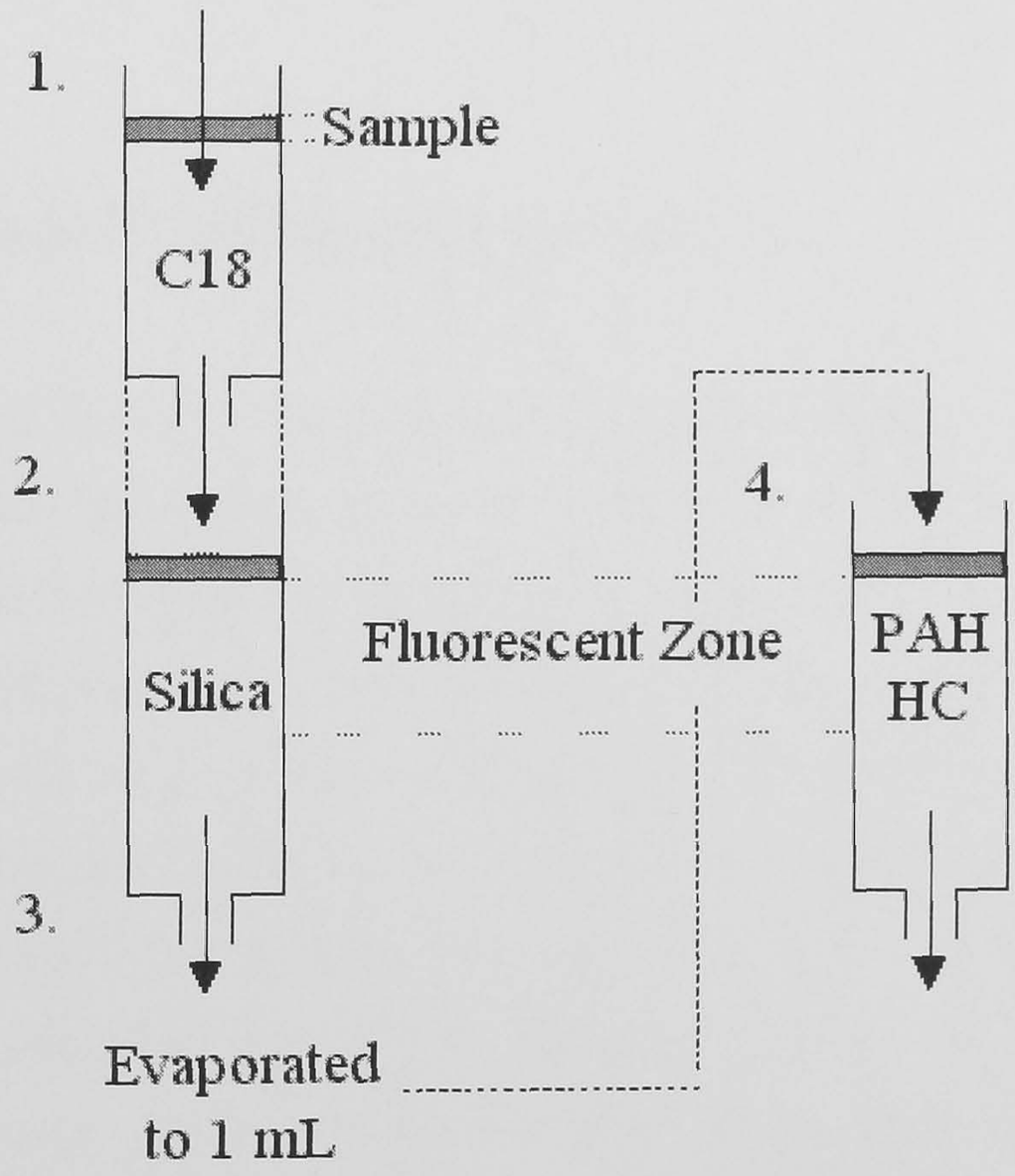


FIGURE 2.1. Outline of the C18/Silica/Isolute extraction of PAHs from transformer Oil. (1) Cyclohexane washes the oil sample through the C18 sorbent into the silica column. (2) C18 is removed and cyclohexane washes the fluorescence through the silica. (3) Acetone elutes the fluorescence, which is concentrated to 1 mL. (4) The fluorescent residue is added to an Isolute PAH HC column, washed with pentane and eluted with acetone for further analysis.

Migration of fluorescent species (including PAHs) was observed using a 366 nm UV light source. The C18 cartridge was discarded and the SiO_2 column washed with 5 mL cyclohexane, which was then discarded. Columns were eluted with 6 mL acetone (3-4 mL min^{-1}) and the eluent evaporated to dryness in a fume cupboard at room temperature. Samples were re-dissolved in 1 mL cyclohexane and applied to a PAH HC Isolute column, preconditioned with 8 mL cyclohexane. The column was washed with 3 mL of pentane, which was discarded, and the final extract was eluted with 6 mL of acetone (3-4 mL min^{-1}) and analysed using GC-MS. All tests were performed 6 times.

2.2.10 Large Scale C18/Silica/Isolute PAH HC

Bakerbond C18 and Silica (10 g) polyethylene columns were purchased from Fisher Scientific Ltd. The C18/ SiO_2 columns were conditioned with cyclohexane (60 mL), which was discarded after use. A weight of 2.5 g transformer oil was loaded onto the C18 column and allowed to penetrate the sorbent for 3 minutes. The C18 column was topped up with 1 mL of cyclohexane as required to prevent drying out of the sorbent. Samples were eluted onto the SiO_2 column with 50 mL cyclohexane under gravity. The C18 column was discarded and the SiO_2 column washed with 60 mL cyclohexane. The SiO_2 column was eluted with 100 mL acetone and the eluent evaporated to 10 mL in a rotary evaporator. As the Isolute PAH HC sorbent was only available as 1 g columns, the sample was split into 1 mL fractions and added to 10 PAH HC Isolute columns preconditioned with 8 mL cyclohexane. Each column was washed with 3 mL pentane (which was discarded) and the final extracts eluted with 6 mL acetone (3-4 mL min^{-1}). These extracts were combined into one sample and evaporated with a rotary evaporator to 25 mL. A small quantity (0.1 mL) was removed for GC-MS analysis whilst the rest of the extract was evaporated to dryness at room temperature in a fume cupboard and reconstituted in 0.5 mL DMSO. This extraction method was repeated twice for each oil and the extract in DMSO combined to give 1 mL of extract from 5 g oil for Ames testing.

2.2.11 Toxicity Testing of Oils and Oil Extracts with *S. Typhimurium*

A 10 µL volume (per well) of a 16 hour *S. typhimurium* culture (Section 2.1.2.1) was added to the wells of a sterile microwell plate along with 250 µL fresh sterile nutrient broth. A 10 µL sample of oil or oil extract was then added. The oil extracts were diluted in DMSO to test for toxicity over a range of doses (3 wells per dose). The microplates were sealed with microwell tape and the *S. typhimurium* was grown for 16 hours at 37°C before absorbance readings were taken at 600 nm with the plate reader.

2.2.12 The Miniscreen Mutagenicity Assay

The miniscreen assay used the same materials and principles as the Ames test, but with smaller multiwell plates with 25 x 100 mm^2 flat bottomed wells instead of petri dishes and therefore used less material and sample.

The procedure was performed as described by Brooks (1995) then carried out with the modifications described by Burke *et al.* (1996). These modifications include the use of a 10 hour culture rather than an overnight culture, a maximum of 10% v/v S-9, and a three day incubation period instead of two days.

A 100 µL volume of 10% v/v S-9 was added directly onto the VB agar plate, along with the 20 µL sample to be tested. The *S. typhimurium* culture was added directly to the top agar and made up 5% v/v of the final top agar solution, which was kept at 42°C. Each well had 0.5 mL of top agar added, the lid replaced and the top agar was allowed to harden before inversion and incubation at 37°C. After 48 hours of growth the colonies were counted as in the Ames test.

2.2.13 Immunoassay Rapid Assay PAH Test Kit

The instructions for the use of both the total and carcinogenic PAH test kits were supplied with the kits, but is briefly outlined below:

1. The oils were diluted in methanol by 10 000 times for the total PAH kit, and by 100 000 for the carcinogenic kit. Further dilution was required for some oils that yielded values at the extreme ends of the dynamic range of the kit. The final dilution was always made in diluent (buffered saline supplied with the kit) so that the final concentration was in 10% v/v methanol.

2. The 250 µL samples were placed in duplicate, in test tubes in the top part of the magnetic rack. The standards were also repeated in duplicate along with a blank (diluent only) and the control solution. The controls and standards were repeated each time the test was performed.

3. A 250 µL volume of PAH analogue labelled with horseradish peroxidase (HRP) (enzyme conjugate) was added to each test tube, followed by 500 µL of magnetic particles coated in anti-PAH antibodies.

4. The test tubes were vortexed, avoiding foaming and incubated for 30 minutes at room temperature. The kit for carcinogenic PAHs was incubated for 20 minutes at room temperature.

5. The bottom part of the rack, which contained the magnets, was added and the test tubes were left for 2 minutes so that the magnetic particles settled to the bottom. The contents was then poured out onto absorbent paper, ensuring the bottom part of the rack was still attached so that the particles remained in the test tubes.

6. The particles were washed with 1 mL de-ionised water/detergent mixture, vortexed and left for 2 minutes, allowing the particles to settle to the bottom of the test

tubes. The contents was then poured onto absorbent paper, ensuring the bottom part of the rack was again attached and the particles remained in the test tubes. This washing test was then repeated.

7. The bottom of the rack was removed and 500 µL hydrogen peroxide and 3,3`,5,5`-tetramethylbenzidine solution (colour solution) was added. The test tubes were vortexed and incubated for 20 minutes at room temperature.

8. A 500 µL volume of sulphuric acid solution was then added to stop the reaction and 200 µL of each test tube was added to the wells of a microtitre plate in duplicate. The microtitre plate was read in a plate reader at 450 nm within 15 minutes of adding the stop solution.

CHAPTER 3.0

RESULTS

MEASURING MUTAGENICITY OF POLYCYLIC AROMATIC HYDROCARBONS IN OILS AND OIL EXTRACTS

3.1 INTRODUCTION

Mutagenicity of transformer oils was determined using the Ames test. The Ames test was chosen as it has been used extensively in the literature and has been recognised by regulatory authorities and the oil industry as a valid mutagenicity test (OECD, 1983; Nynas, 1999).

The aim of the work described in this chapter was to determine the mutagenicity of the oil sample, and also oil aromatic fractions, thought to be the source of most risk (Tolbert, 1997). By comparing oil and aromatic mutagenicity, the aromatic contribution (and more specifically PAH contribution) to whole oil mutagenicity can be ascertained. A transformer may contain up to 100 000 litres of oil and therefore the health risk to National Grid Company Plc employees, and the environmental risk through leakage is significant. A measure of whole oil and PAH mutagenicity would indicate whether certain oils should be avoided to reduce the risk. It would also ascertain whether removing PAHs would be a suitable measure, considering the beneficial properties PAHs bring to the oil (Section 1.1.2).

In order to measure oil mutagenicity, a number of issues required consideration. The hydrophobic properties of the oil did not lend themselves to testing in the aqueous environment of the Ames test. As oil is a complex mix of components, mutagenicity may be increased or decreased by synergistic or antagonistic effects from other components. This may affect the bacteria and the activation enzymes present in the Ames test. Oil interference effects were therefore investigated.

The aromatic fraction of the oil was extracted by various liquid-liquid and solid phase extractions in order to determine the optimum method of oil preparation and hence compatibility with the Ames test. As stated by Stang (1993) the fraction extracted by liquid-liquid methods contains all DMSO soluble components of the oil and will contain substances other than polyaromatic components (PAHs). Results were therefore analysed in order to distinguish PAH mutagenicity from other potentially

mutagenic sources. The dose of oil extract for Ames testing was given as milligrams of oil extracted (as opposed to milligrams of oil extract) per plate, as is the convention in the literature (Herman *et al.,* 1981; Granella *et al.,* 1991, 1995; Brooks *et al.,* 1995) to allow a better comparison of whole oil mutagenicity results with oil extract results.

Four oils were tested to determine how mutagenicity differed with PAH content. White oil, a highly refined mineral oil, was used as a 'blank' as it contained little or no PAHs along with transformer oil 4, thought to contain a low PAH level according to IP 346 % w/w data. Oil 8 was used as the high PAH content oil, and Nytro-10GBN (N10GBN) was tested as it was thought to have an intermediate PAH level and is the most widely used oil in transformers by the National Grid Company Plc.

3.2 AMES TESTING OF EPA 16 PRIORITY PAH STANDARD

Before the oils could be tested for mutagenicity, the *Salmonella typhimurium* strain TA98 had to be tested with PAH standards to ensure it was the correct strain to use for PAH mutagenicity. Although this strain has been used in the literature for determining PAH mutagenicity in oil (Blackburn *et al.,* 1984; 1986; Brooks *et al.*, 1995; Granella *et al.*, 1991; 1995) the available EPA 16 priority PAHs were tested to ensure that the results obtained agreed with the findings in the literature. As the International Agency for Research on Cancer (IARC, 2002) has the most comprehensive data collection on PAH mutagenicity, their findings were used for comparison.

3.2.1 Effects of DMSO on *S. Typhimurium*

In order to assess the oil or oil extracts with the Ames test, the oil was added to a polar solvent that was miscible with the oil but not toxic to *S. typhimurium*. Dimethyl sulphoxide (DMSO) was the solvent used by Maron and Ames (1983) as most other organic solvents are toxic to *S. typhimurium* (Salamone *et al.,* 1979). However, it was important to establish the amount of DMSO that could be added without interfering with the assay, as the more that could be added, the greater the amount of sample that

could be tested. Increased quantities of DMSO were added to the *S. typhimurium* assay to determine the toxic effects of DMSO.

TABLE 3.1. Effect of increased volume of DMSO as a solvent carrier in the Ames test.

DMSO	*0 mL*	*0.1 mL*	*0.2 mL*	*0.3ml*	*0.5ml*
Mean Number of Revertants	43	43	24	4	0
± SD	3.5	1.5	2.3	2.7	0

It was clear from the results in Table 3.1 that 0.1 mL DMSO would be appropriate for Ames testing as all other quantities lead to a reduction in revertant number. This meant that the limiting factor in testing oil samples or extracts was the amount of test substance that was soluble in 0.6 mL of DMSO (the volume required for testing each sample with and without S-9). For the purpose of repeat testing, the test substances were reconstituted in 1 mL DMSO, leaving an extra 0.4 mL for diluting to lesser doses.

3.2.1.1 Mutagenicity of Individual EPA 16 Priority PAHs

PAHs, considered to be priority pollutants by the EPA, were tested over a range of 5 doses. Each dose was diluted 10-fold to ensure that a large dose range was assessed. As mutagenicity of the control mutagen benzo[a]pyrene was observed at 5 $\mu g\ plate^{-1}$, and 2-amino anthracene at 2 $\mu g\ plate^{-1}$, PAHs showing a doubling of revertants at similar doses were considered mutagenic (Jackson, 1986). However, the range of doses tested extended from 0.5 to 5000 $\mu g\ plate^{-1}$ to ensure that less potent mutagens were also noted (OECD, 1983). Not all of the EPA 16 priority PAHs were tested. Those that were well documented as carcinogens and were unlikely to be present in the oil according to National Grid Company Plc analysis (Pahlavanpour, and Wilson, 1999)

were not tested. These were benzo[b]fluoranthene, dibenz[a,h]anthracene and indeno[1,2,3-c,d]pyrene.

Results are summarised in Table 3.2. Each sample was tested with and without 10% v/v S-9 in triplicate on 2 separate occasions to ensure repeatability (total of 12 plates per sample). Only the results on addition of S-9 are shown since no mutagenicity was observed for any of the PAHs in the absence of S-9. Toxic effects, observed as a lack of background bacterial lawn or a reduction in revertant number compared to the spontaneous background rate, were also recorded.

TABLE 3.2. Mutagenicity of PAH standards tested with the Ames test. Up to 5 mg plate^{-1} was used. A minus sign denotes no mutagenic/toxic effect; a plus sign denotes mutagenic/toxic effect.

Name of PAH	*Mutagenicity according to the findings of the IARC (1983)*	*Mutagenicity with 10% v/v S-9 from the Ames test*	*Toxicity from the Ames test*
Naphthalene	-	-	-
Acenaphthylene	-	-	-
Acenaphthene	-	-	+
Fluorene	-	-	-
Phenanthrene	-	-	+
Anthracene	-	-	-
Fluoranthene	-	-	+
Pyrene	-	-	-
Benzo(a)anthracene	+	+	+
Chrysene	+	-	-
Benzo(b)fluoranthene	+	N/A	N/A
Benzo(k)fluoranthene	+	+	-
Benzo(a)pyrene	+	+	-
Indeno(1,2,3-c,d)pyrene	+	N/A	N/A
Dibenz(a,h)anthracene	+	N/A	N/A
Benzo(g,h,i)perylene	-	+	-

N/A = not IARC recognised carcinogen, not tested

The findings of the Ames test agree with those compiled by the IARC except for chrysene, which was found to be non-mutagenic in the Ames test, but classed as a mutagen by the IARC. The mutagenic dose response for chrysene is shown in Figure 3.1. Although no doubling of the spontaneous revertants was observed even at the highest dose (5 mg plate^{-1}) there was still an increase in the number of revertants compared to the response at 0 mg plate^{-1}. According to the protocols published since the development of this test in 1975, mutagenicity is only established if at least a doubling of revertants is observed with a dose-related response (Jackson and Pertel, 1986). For this reason, chrysene cannot be classed as a mutagen on the results of the Ames test. However, it cannot be considered a non-mutagen, due to the borderline results obtained and the conclusion of the IARC. However, such discrepancies for chrysene have been found in the literature (Basler *et al,* 1977; McCann *et al*, 1975) and are discussed further in Section 7.2.1. Therefore, strain TA98 was deemed appropriate for testing PAHs.

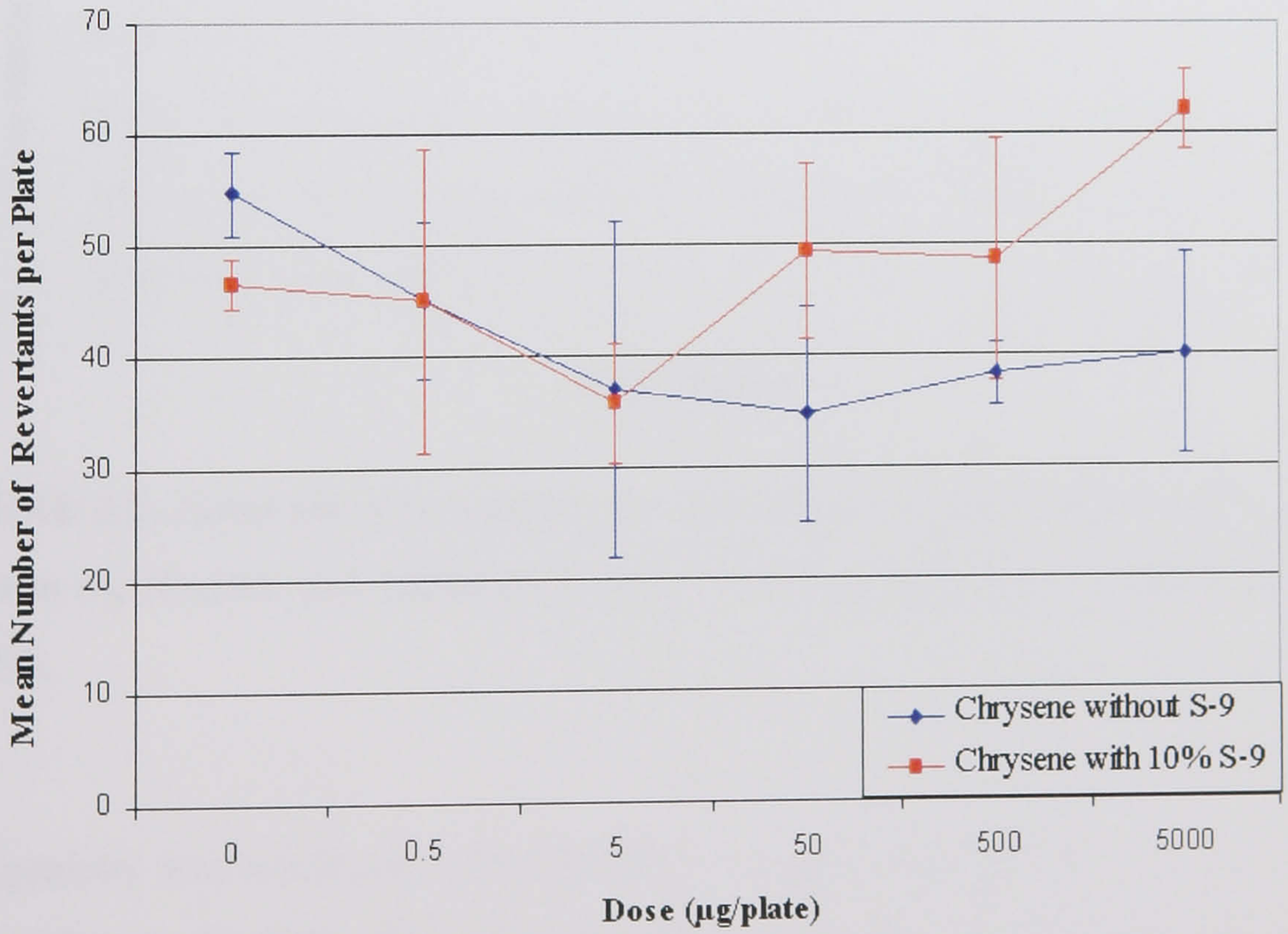


FIGURE 3.1. Ames test revertants on the addition of chrysene in the absence and presence of 10% v/v S-9 solution (n = 3). %CV ranges from 5-40%.

3.2.1.2 Mutagenicity of EPA 16 Priority PAH Mixture

In addition to testing the EPA 16 priority PAHs separately, a mixture composed of the same amount of each PAH was tested over a range of 5 doses. In accordance with the work of Blackburn *et al.* (1984) a greater amount of S-9 (80% v/v) was used. The highest dose added was 2 mg plate^{-1} (per PAH) due to availability limits of the EPA 16 priority PAHs. Results are shown in Figure 3.2.

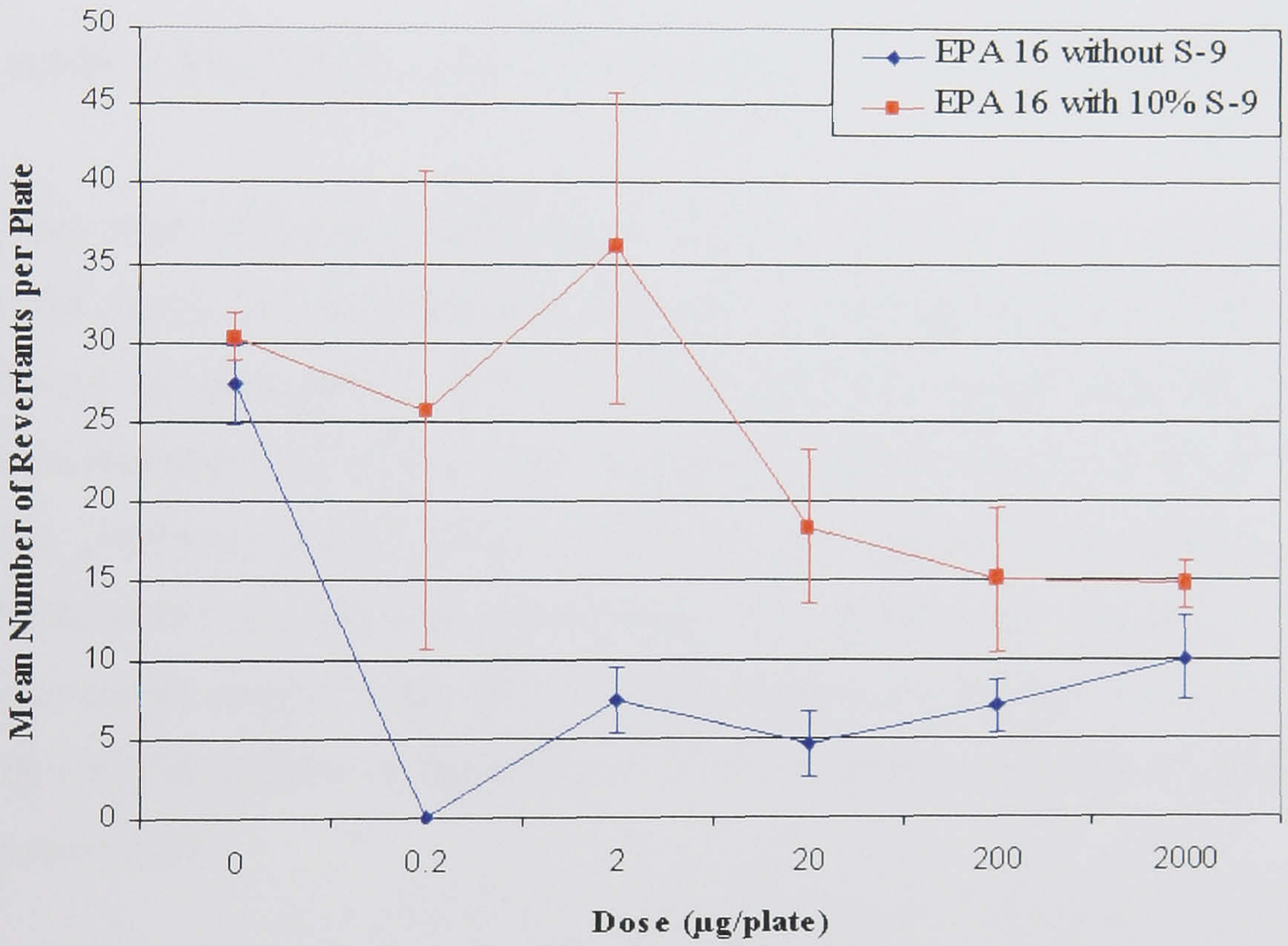


FIGURE 3.2. Ames test revertants on the addition of a mixture of the EPA 16 priority PAHs in the absence and presence of 10% v/v S-9 solution (n = 3). %CV ranges from 10-96%.

Mutagenicity was not observed for the EPA 16 priority PAHs. However, a toxic effect was evident in the form of a reduced number of revertants compared to the negative control (0 mg plate^{-1}), on the plates without S-9. The number of revertants observed with S-9 present was not as reduced, indicating that a mutagenic increase in revertants was counteracting the toxic decrease in revertants (Haroun and Ames, 1981). Up to a

dose of 2 μg plate^{-1} the number of revertants increases suggesting an increase in mutagenicity. However, at 20 μg plate^{-1} and above, the toxicity overcomes any mutagenic effects and a decrease in revertant number is prevalent.

These results illustrated that a mixture of PAHs presents a complex interplay of mutagenic and toxic effects. The toxicity of the oils must therefore be established to ensure it was not interfering with mutagenicity measurements. Toxicity is further discussed in Section 5.8.

3.3 AMES TESTING OF WHOLE OIL

The first stage of testing transformer oil for mutagenicity was to test the oil directly with the Ames test, to determine whole oil mutagenicity. In early papers reporting studies of oil mutagenicity with the Ames test, the oil was either (a) added to the bacteria directly or (b) added with a detergent present (Tween 80) to make the oil more soluble (Hermann *et al.*, 1980). A 10% v/v S-9 solution was used in each case. Differences in response, with and without S-9 would indicate whether PAHs were the only mutagens present in the oil. If PAHs were the only mutagens, a positive response would only be evident in the presence of S-9 (indirect mutagenicity). Both methods were examined.

3.3.1 Direct Addition of Oil

The main concern with directly adding the oil to the Ames test was that the oil was predominantly hydrophobic and therefore may not mix well with the aqueous components of the Ames test, thus giving inconsistent results.

TABLE 3.3. Ames test revertants on the addition of pure oil (0.1 mL) in the presence and absence of 10% v/v S-9 solution (n = 3).

Components of Ames Test Plate	*Oil 8*	*Oil 8*	*Oil N10GBN*	*Oil N10GBN*	*Oil 4*	*Oil 4*
S-9 (10% v/v) (mL)	0.0	0.5	0.0	0.5	0.0	0.5
Mean No. of Revertants plate^{-1}	40	43	45	41	40	45
± SD	17	15	13	13	15	13
%CV	43	34	28	31	37	28

On adding pure oil to the Ames test, no mutagenicity or toxicity was observed (Table 3.3). However, the coefficient of variance (%CV) varied between 27-43% for all the oils, suggesting incompatibility between the oil and water matrices. The assay was not sufficiently repeatable to give conclusive results. Making the oil soluble with Tween 80 was considered a means of overcoming this problem.

3.3.2 Oil with Tween 80

A 200 µL volume of oil was made soluble in 200 µL Tween 80 as described by Hermann *et al.* (1980). De-ionised water was added dropwise while stirring, until a final volume of 2 mL was reached (10% v/v Tween 80). The results for oil 8 are shown in Table 3.4.

The addition of Tween 80 improved the repeatability of the method as the %CV values were reduced to between 3-28% (as opposed to 27-43% for the pure oil). No doubling of the spontaneous reversion rate was observed at any dose and no indication of toxicity was observed. Due to the complex mixture of components that make up the oil however, it was possible that there were antagonistic components reducing mutagenicity that could not as yet be accounted for. Whole oil mutagenicity is further discussed in Section 7.2.2.

TABLE 3.4. Ames test revertants on the addition of oil 8 made soluble with Tween 80 in the absence and presence of 10% v/v S-9 (n = 3).

Oil (mg plate^{-1})	*Oil 8/Tween 80 without S-9*	*± SD*	*%CV*	*Oil 8/Tween 80 with 10% S-9*	*± SD*	*%CV*
0	41	1.4	3	41	1.4	3
0.000615	38	3.5	9	40	2.6	7
0.00123	43	9.0	21	44	10.7	24
0.00615	51	3.2	6	29	2.6	9
0.0123	39	7.5	19	52	7.2	14
0.0615	37	4.3	12	58	16.0	28
0.123	50	14.4	29	53	5.5	10
0.615	47	6.8	14	54	3.5	6
1.23	46	11.5	25	52	5.7	11
6.15	33	8.5	26	28	8.6	30
12.3	36	5.5	15	32	11.2	35

By investigating the mutagenicity of the aromatic fraction of the oil, (Section 3.4.1) thought to be the source of mutagenicity (McKee *et al.,* 1989; Järvholm and Easton, 1990; Brooks *et al.,* 1995; Granella *et al.,* 1995) antagonistic effects may be revealed if the oil contained mutagens. If no mutagenicity were observed in the aromatic fraction however, it would remain unclear if this result were due to a lack of mutagen or an antagonistic effect. For this reason further examination was required. By adding oil to a known mutagenic PAH such as benzo[a]pyrene and 2-amino anthracene, antagonism could be unequivocally established.

3.3.3 Investigation of Whole Oil Interference on the Ames Test

To determine if the oil would affect the mutagenicity of PAHs, 0.1 mL whole oil was added to the bacteria with 0.1 mL Benzo[a]pyrene (50 μg mL^{-1}) or 2-amino anthracene (20 μg mL^{-1}). Oil 8 was tested as it had the highest aromatic loading

according to IP 346 % w/w data, oil 4 as it contained the lowest and white oil was tested as a blank.

Results in Table 3.5 show that the presence of oil reduced the number of revertants by between 16.6-33% for plates with benzo[a]pyrene and 11.5-25% for plates with 2-amino anthracene. The %CV of benzo[a]pyrene was ~7% and 2-amino anthracene was ~5% which was low and meant that a reduction of > 7% may be significant.

TABLE 3.5. Effects on revertant number of adding whole oil to mutagens benzo[a]pyrene and 2-amino anthracene with 10% v/v S-9 (n = 3).

Sample on Plate	*Mean Number of revertants*	*± SD*	*%CV*	*Reduction in Revertants from Mutagen on Exposure to Oil (%)*	*One-Sided t-Test (t) **
Benzo[a]pyrene (B[a]P) only	196	34.1	17	N/A	N/A
White oil and B[a]P	163	20.8	13	17	-2.7
Oil 4 and B[a]P	141	15.5	11	28	-6.1
Oil 8 and B[a]P	131	2.6	2	33	-43
2-amino anthracene (2-aa) only	729	37.1	5	N/A	N/A
White oil and 2-aa	645	83.3	13	12	-1.7
Oil 4 and 2-aa	554	47.7	9	24	-6.3
Oil 8 and 2-aa	547	28.5	5	25	-11

**Critical Value t3 = -2.35 (P = 0.05)*

A t-test was used to determine significance. The null hypothesis (n_0) was that there was no difference in revertant number on the addition of oil except for that from random error. A one-sided t-test was used, and the results in Table 3.5 were given a negative value, as only a decrease in revertants was of interest. The critical t-value for three repeats (t_3) was –2.35 and any t-value of greater negative value was considered significant (the null hypothesis was rejected).

All values in Table 3.5 showed a significant reduction in the number of revertants except for white oil with 2-amino anthracene. White oil with benzo[a]pyrene was considered significant at the 95% level, but with a difference of -0.36 which was small compared to the other oils, which differed by at least –3.78.

The largest decrease in revertants was observed with oil 8, spiked with either benzo[a]pyrene or 2-amino anthracene whilst the smallest reduction in revertant number was observed with white oil (17% for benzo[a]pyrene and 12% for 2-amino anthracene). It was therefore possible that the PAHs present in oil 8 interfered with benzo[a]pyrene or 2-amino anthracene mutagenicity. The t-value differences also increased with the PAH content of the oil, suggesting that the other PAH species may play a part in inhibition. This is further discussed in Sections 3.8, 5.7 and 7.5.1.

The inhibition observed here may interfere with borderline or weakly positive Ames test results. In the case of oil 8, the number of revertants produced at the highest dose with Tween 80 was 36 (Table 3.4). The greatest inhibition of revertants recorded was 32% for oil 8, but increasing the number of Ames test revertants by 32% yields a value of 47.5 which does not constitute a doubling over the zero dose response. It could be concluded in this case that inhibition did not lead to a false negative result. It does illustrate however, that measuring mutagenicity of a complex sample may prove inconclusive until the inhibition from other components in the sample is established.

The next stage was to determine what part of the Ames test assay was affected by the oil matrix components. One possibility was that the oil inhibited the growth of the bacteria, or inhibited bacterial uptake of the mutagen. Alternatively, the oil could have been affecting enzyme activity, reducing PAH activation. It was also possible that both the enzyme and bacteria were being affected. Under the current experimental protocol, it was not possible to isolate possible affects on bacteria from the enzyme activity in the Ames test since all components were incorporated into the top agar. A completely different approach was therefore required.

3.3.3.1 The Impact of Oil on S-9 Activity

To determine if the oil affected enzyme activity, 10% v/v S-9 was incubated for 20 minutes with various amounts of oil extract and incorporated into the Ames test to determine if its activity had been altered. After incubation with the oil, the S-9 was removed by centrifuging the mix for 15 minutes. Centrifugation has been suggested as a way of removing the activation enzymes from the mix of other proteins in the S-9 (Brooke *et al.,* 1996). Once the pellet containing the activation enzymes was collected it was added to the Ames plates with *S. typhimurium* and Benzo[a]pyrene. It was thought that if component in the oil acted to inactivate the enzyme, it would be observed on the Ames plates by a reduction in revertant number.

However, the centrifuged S-9 controls showed that even without the oil present the number of revertants was significantly reduced. It was possible that the centrifugation process either interfered with enzymes activity, or more S-9 was required to make up for losses incurred due to incomplete separation. However, repeating the experiment with more S-9 and with an extended centrifugation period showed no improvement, so an alternative method was developed.

3.3.3.2 The Impact of Oil on S-9 Activity and S. Typhimurium with Liquid Ames Test

A liquid media version of the Ames test was investigated to alleviate the difficulty of determining the oil's affect on bacteria and enzymes separately. No known protocol has been reported in the literature since no investigation of this particular problem had been performed. The protocol used is outlined in Section 2.2.5.

The method was performed in 2 stages (1) incubation of the S-9 with the mutagen and *S. typhimurium*, and (2) isolation of the *S. typhimurium* from the mixture and growth in fresh media devoid of histidine. If the bacteria had not reverted, no growth would be observed in the second stage. This would suggest that the oil affected the S-9. Oil was

then added to the fresh media in the second step to determine if the oil interfered with the growth of the *S. typhimurium*. Controls were run accordingly, to determine changes at each stage, and are included in Table 3.6.

TABLE 3.6. Effects of pure oil on S-9 activity and *S. typhimurium* growth (n = 12).

Amount of Oil Added During Incubation of Bacteria, S9 and B[a]p	*Amount of Oil Added During Growth in Microtitre Plate*	*Amount of Oil Added After Growth in Microtitre Plate*[§]	*Mean Absorbance*	*± SD (n = 12)*	*One-Sided T-Test (t)**
0	0	10	0.87	0.035	-
0	0	25	0.89	0.034	-
0	10	0	0.53	0.022	-52
0	25	0	0.28	0.040	-52
10	0	10	0.83	0.016	-7
25	0	25	0.85	0.035	-4

** Critical Value t_{12} = -1.78 (P = 0.05)*

§ Oil added at this stage to account for the effect of oil presence on absorbance readings

It can be seen in Table 3.6 that bacteria growth was most significantly affected. The greatest absorbance drop was in samples where oil was added during the second stage of testing where only *S. typhimurium* was present. This result suggests that other components in the oil extract were preventing the PAHs from entering the bacterial cells. The t-test values suggested however, that oil added during the first stage also significantly decreased the observed absorbance, as the t-values were less than the critical t value of –1.78. This result was expected, as *S. typhimurium* was also present during this stage. This did not rule out issues concerning enzyme inhibition, but as this decrease in absorbance became greater when oil was added to the second stage of growth, it at least suggested that *S. typhimurium* was affected by the presence of oil.

The high level of mutagenicity engendered by benzo[a]pyrene and 2-amino anthracene even with oil present, suggested that the oil itself does not contain mutagens, as naturally occurring mutagens would have been revealed in previous experiments (Section 3.3). However, the benzo[a]pyrene and 2-amino anthracene added to the Ames test were not intrinsic components of the oil, and it was therefore postulated that mutagenicity of PAHs within the oil matrix would be affected differently.

In addition, it was possible that the oil contained such low amounts of mutagenic PAH that a larger volume of oil was required before a mutagenic response was observed. More oil could not be added to the test system, as it would significantly hinder bacterial growth in an undesirable oily environment. As shown from the previous experiment, the presence of the oil seemed to affect bacterial growth, so the effects of the oily matrix required reduction. It was postulated that concentrating the sample, reducing the oil matrix and hence removing antagonistic effects, could be achieved by extracting and testing the aromatic fraction of the oil, thought to contain the mutagens (Brooks *et al.*, 1995; Granella *et al.*, 1995).

3.4 AMES TESTING OF OIL EXTRACTED BY LIQUID-LIQUID SEPARATION

The IP 346 % w/w method is the oil industry standard for the estimation of the polyaromatic content of an oil (Section 2.2.3.1) although it is known to overestimate polyaromatic content (as with all liquid-liquid extractions) by extracting all aromatics into DMSO (Stang, 1993; 1999). For this reason it was considered pertinent to test such extracts for mutagenicity. Since the IP 346 % w/w method is a time consuming and laborious multi-step method, the simpler Grimmer method was also performed and extracts tested for mutagenicity (Section 2.2.3.2). The Grimmer method is also a liquid-liquid extraction (LLE) method, and can be used in the same way as IP 346 to estimate PAH content. Both methods were performed 4 times, to determine repeatability (Table 3.7). It was clear that the IP 346 % w/w values for oil 4 and 8 were

different to those provided by the National Grid Company Plc (Table 2.1). This difference may be due to the number of steps involved in the procedure, which lack precision due to the crude separation process of LLE. The Grimmer data was closer in value to those in Table 2.1 and showed similar trends in PAH content. Lack of repeatability may affect Ames test data. For these reasons, the 4 extracts of each oil were tested for mutagenicity to determine variation in extraction.

TABLE 3.7. Percentage PAH extracted using the IP 346 and Grimmer methods (n = 4).

OIL	*Mean IP 346 value*	*± SD*	*%CV*	*Mean Grimmer value*	*± SD*	*%CV*
Oil 4	4.1	2.1	52	1.9	0.7	38
Oil 8	4.5	0.8	19	5.7	1.2	21
N10GBN	1.7	0.5	29	2.6	1.4	53

3.4.1 IP 346 Extraction and Grimmer Extraction

A 10% v/v S-9 mixture was used for preliminary Grimmer and IP 346 extract analysis to conserve limited supplies of S-9 and determine if a lower concentration of S-9 would indicated mutagenicity. Eighty percent S-9 was used when the optimum extraction method was established. In both cases up to 5 g of oil was tested, as the extract of more than 5 g was not soluble in 1 mL DMSO for Ames testing (discussed in Section 1.4.1.6). The revertant numbers found on individual plates are compiled for IP 346 and Grimmer extracts in Appendix C.

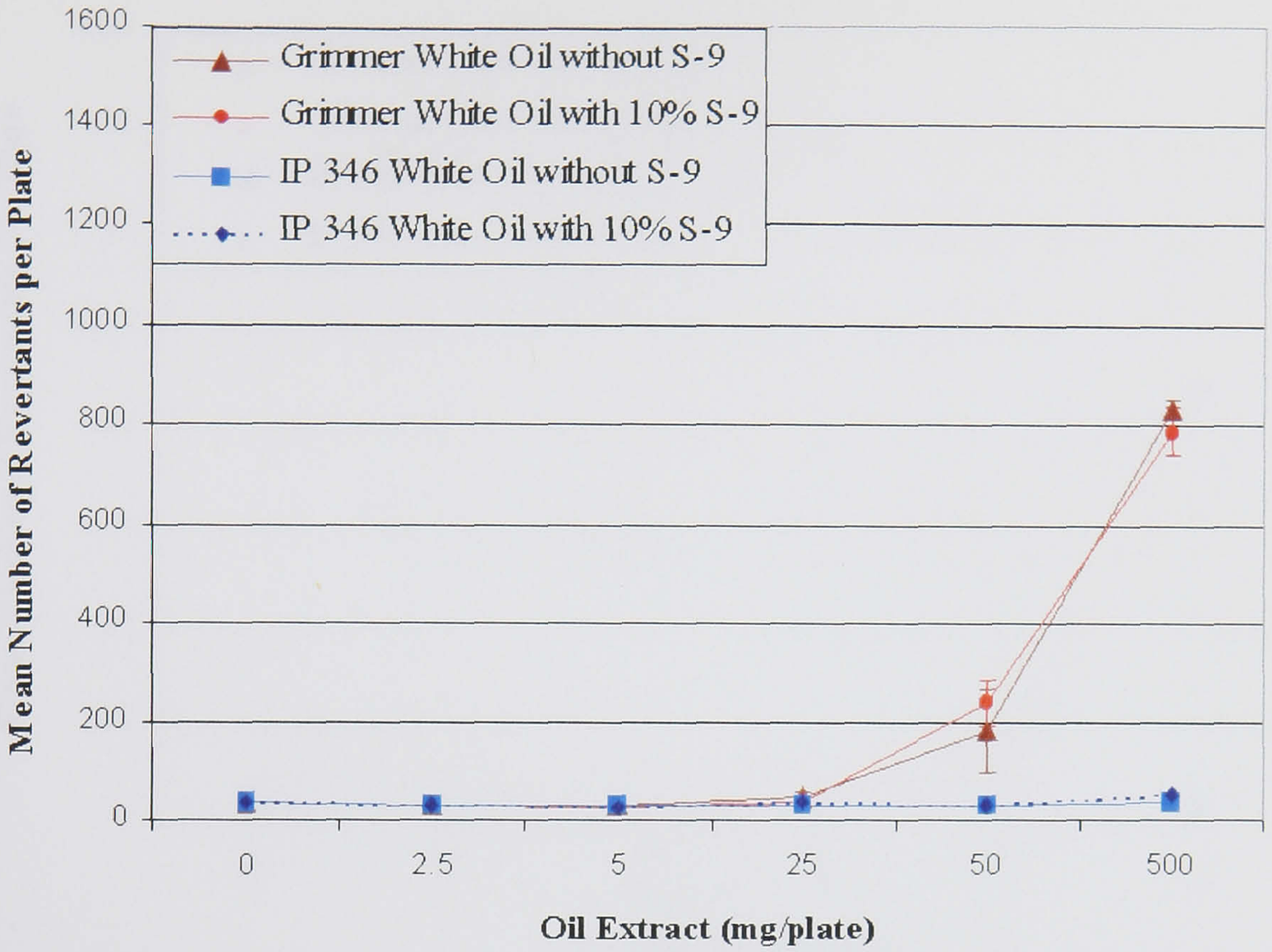


FIGURE 3.3. Ames test revertants on the addition of liquid-liquid extracts of white oil in the absence and presence of 10% v/v S-9 (n = 3). %CV ranges from 2.9-18.7% for IP 346 and 2.3-16% for Grimmer extracts.

White oil (Figure 3.3) and oil 4 (Figure 3.4) showed similar mutagenicity with and without S-9 for the Grimmer extracts. This suggested that the addition of S-9 did not activate more mutagens and that the mutagenicity of the oil was due to components other than PAHs at extract loadings of 50 mg plate^{-1} or greater. The direct mutagens may be due to simple aromatic compounds that are known to be extracted along with polyaromatics in liquid-liquid extractions (Stang, 1993; 1999). However, no mutagenicity was observed for any of the IP 346 extracts with or without S-9, so only Grimmer results will be discussed. IP 346 extract mutagenicity is further discussed in Section 7.2.3.2.

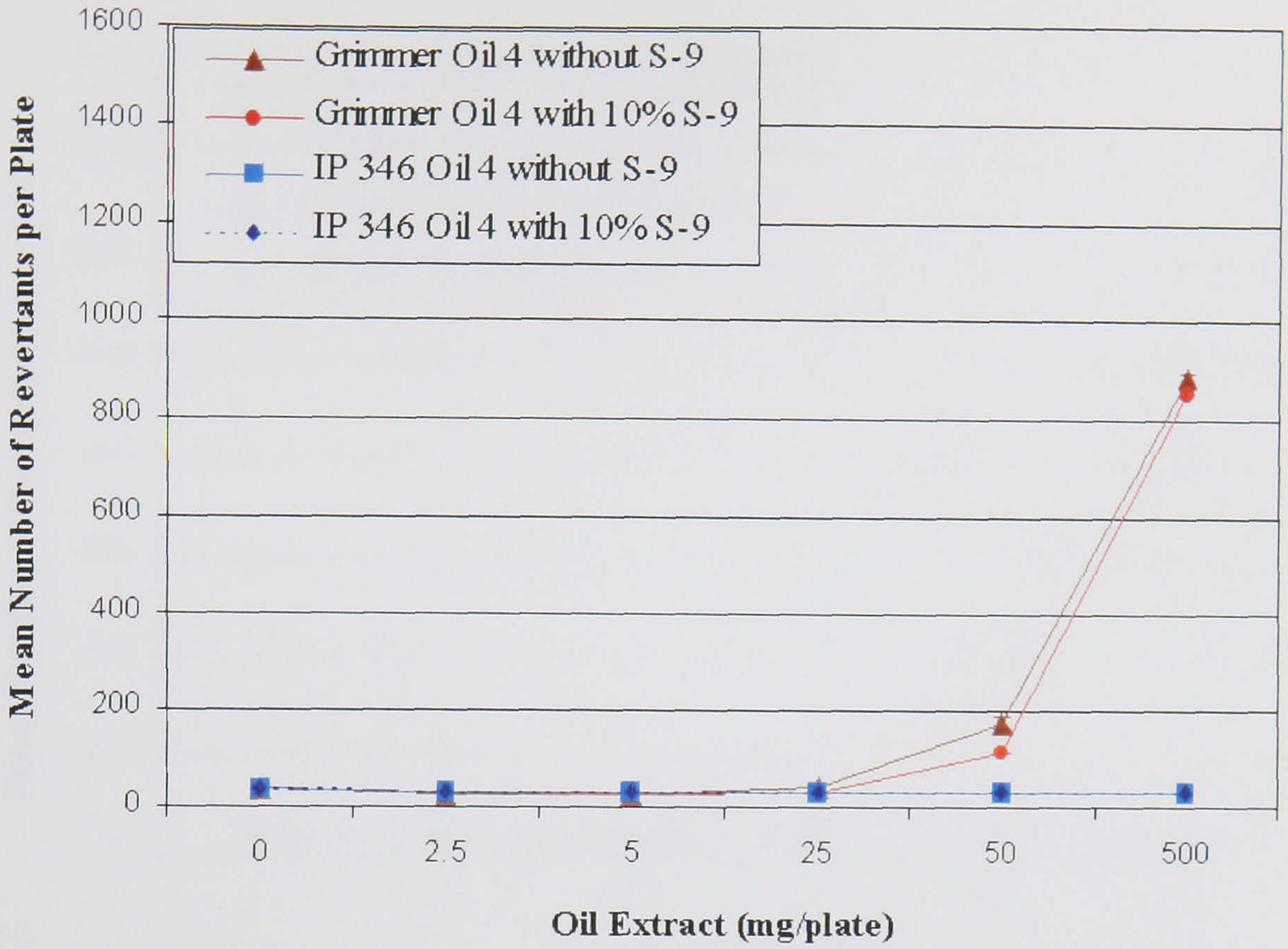


FIGURE 3.4. Ames test revertants on the addition of liquid-liquid extracts of oil 4 in the absence and presence of 10% v/v S-9 (n = 3). %CV ranges from 3.2-11.2% for IP 346 and 3.2-12.5% for Grimmer Extracts.

Oil Nytro-10GBN (Figure 3.5) showed direct mutagenicity at 50 mg plate^{-1} or above. Indirect mutagenicity was not observed until 500 mg plate^{-1} but again only with the Grimmer extract. The number of revertants at 500 mg plate^{-1} increased from 837 without S-9 to 1037 with S-9, which suggested an accumulation of both direct and indirect mutagens. However, as the indirect mutagens were only observed at the highest available dose, the Grimmer extract of Nytro-10GBN was investigated further with the use of 80% v/v S-9 (Section 3.4.2).

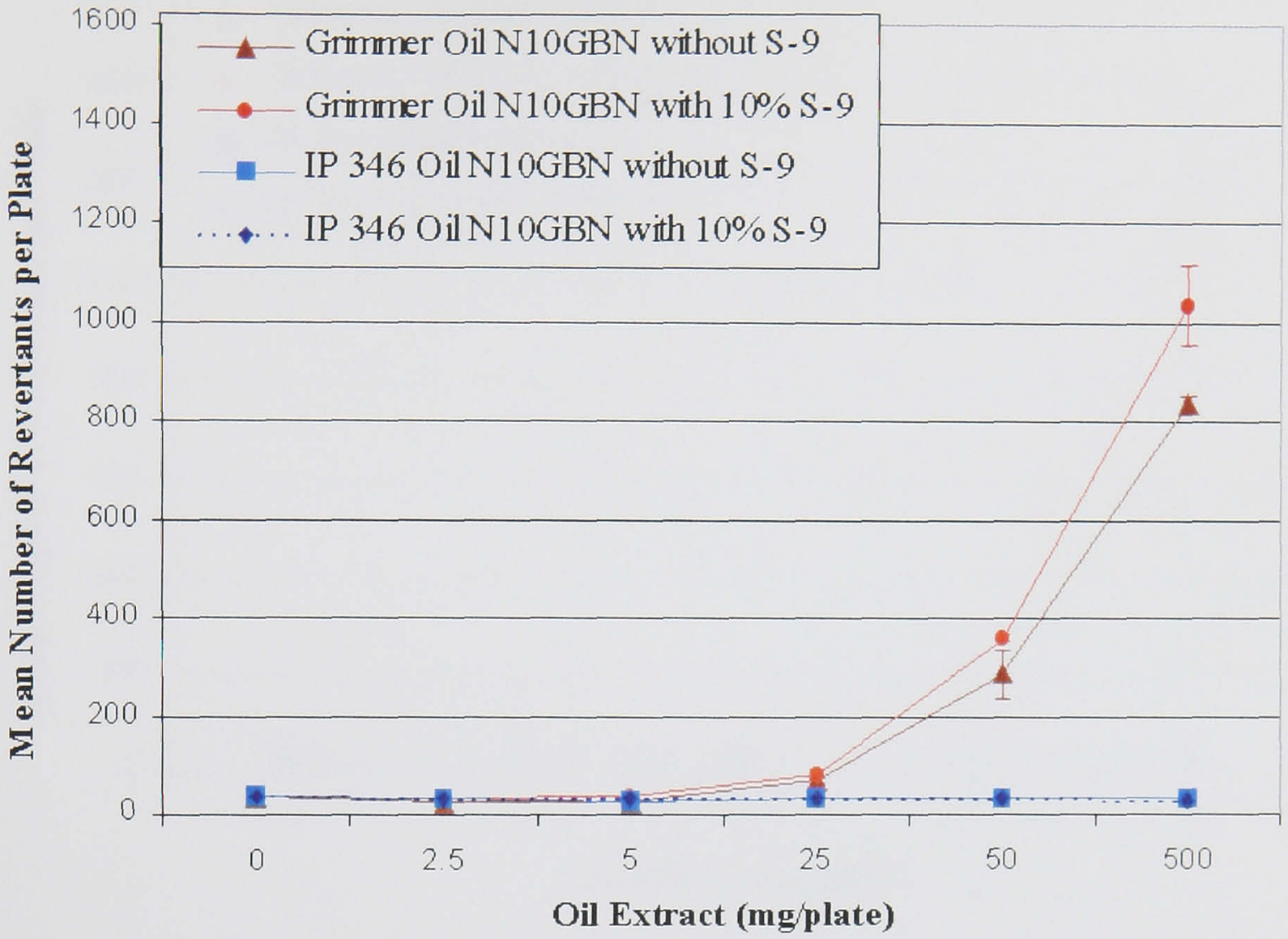


FIGURE 3.5. Ames test revertants on the addition of liquid-liquid extracts of oil Nytro-10GBN in the absence and presence of 10% v/v S-9 (n = 3). %CV ranges from 1.6-9.5% for IP 346 and 1.7-17.9% for Grimmer Extracts.

For oil 8 (Figure 3.6), which was known to have the highest aromatic content (IP 346 % w/w data) only clear evidence of direct mutagenicity (1200 revertants) was observed for the Grimmer extract. This might have been due to the presence of an excess of PAHs in oil 8 competing for the S-9 enzymes and was therefore investigated further using 80% v/v S-9 (section 3.4.2). Results are further discussed in section 7.2.3.

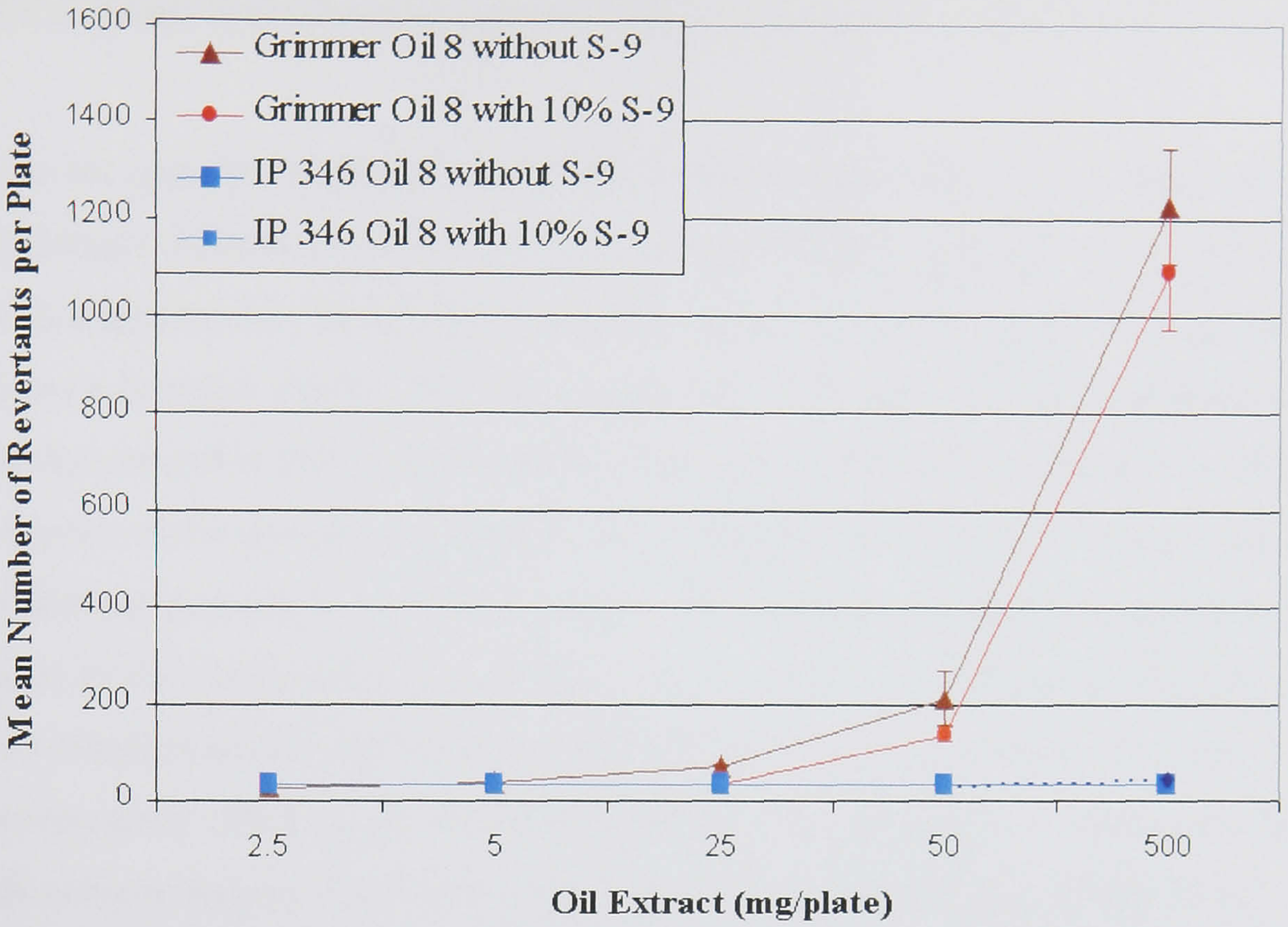


FIGURE 3.6. Ames test revertants on the addition of liquid-liquid extracts of oil 8 in the absence and presence of 10% v/v S-9 (n =3). %CV ranges from 1.6-12.9% for IP 346 and 3.2-27.2% for Grimmer Extracts.

The Grimmer extraction was performed a further 3 times and tested with the Ames test on each occasion. LLE proved difficult to repeat with precision but the Ames test results did not alter each time a new extract of the same oil was used. The variation in aromatic content therefore had no effect on the mutagenicity of the fraction. Hence the results suggested that, although the amount of mutagen might vary with extraction, the threshold level required for mutagenicity was present in all extracts.

3.4.1.1 Total Ion Chromatogram (TIC) of Grimmer and IP 346 Extracts

It was not possible in the context of this work to determine how the Grimmer and IP 346 extracts differed in composition as both gave highly complex GC-MS data (Figure 3.7). It might be that the Grimmer method, due to a reduced number of stages, resulted in a more complex extract, and the mutagenicity observed was due to components that were not present in the IP 346 extracts. However, it was still not possible to determine the source of the mutagenicity due to the remaining complexity of both extracts, and it was also not possible to conclusively relate mutagenicity as observed with S-9 to PAH content as the extract may contain other species of indirect mutagens. The cause of the direct mutagenicity could not be attributed for the same reason. The selective ion chromatogram (SIC) of the oil extracts were also performed to specifically quantify PAH content, but no PAHs were found other than fluorene and phenanthrene. As the %CV values ranged from 59-68% due to the nature of the sample matrix and extraction methods, this data was discarded and is not discussed here.

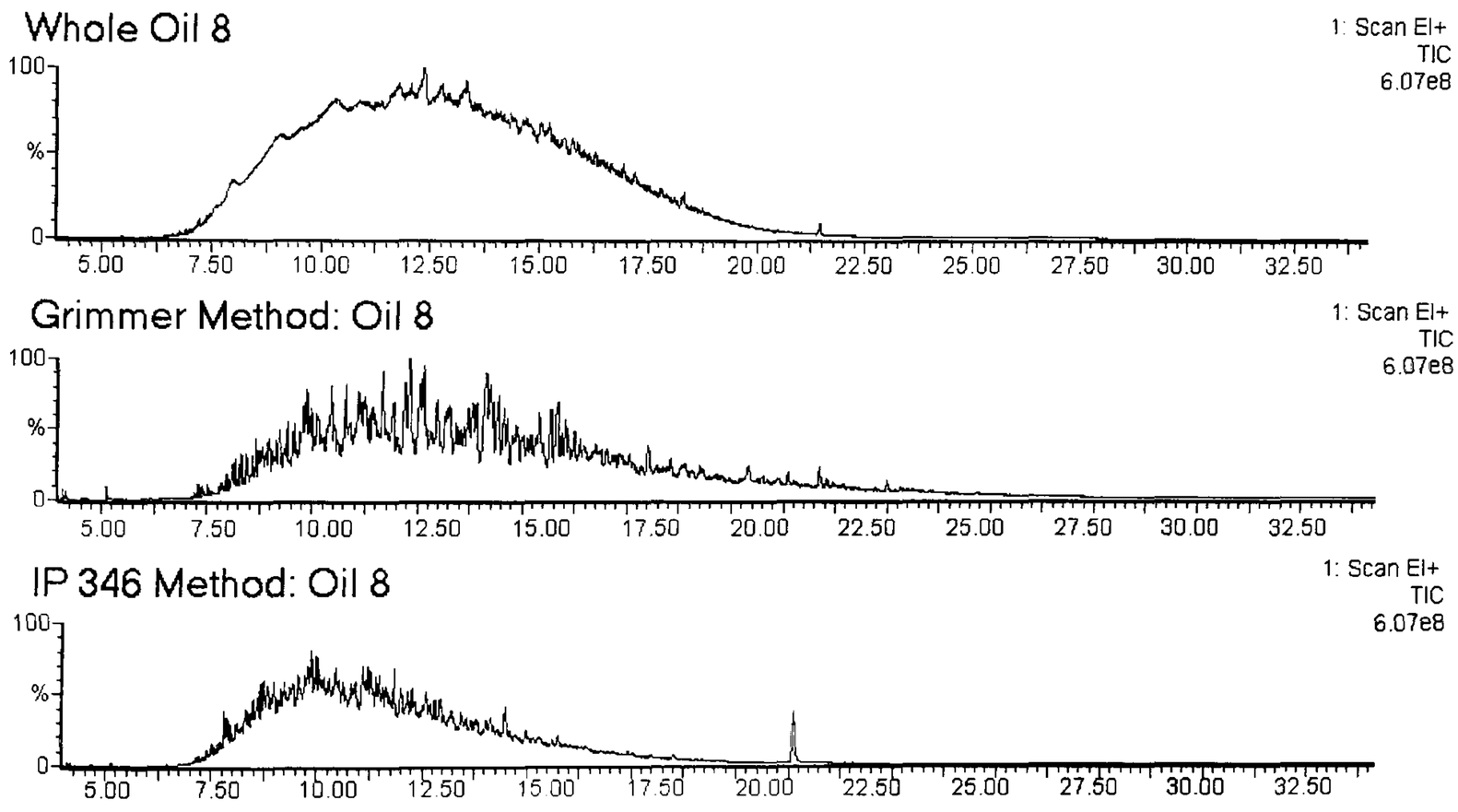


FIGURE 3.7. The total ion chromatograms (TIC) of oil 8 before extraction, after Grimmer extraction and after IP 346 extraction.

3.4.2 The Grimmer Method at 80% S-9

An 80% v/v S-9 mix was used in the Ames test to increase the activation of PAHs in the oil extracts. The number of revertants observed after the addition of 80% v/v S-9 did not significantly change for the Grimmer extract of white oil when compared to the result observed without S-9 (Figure 3.3). This also applied to oil 4 when comparing Figure 3.8 with data recorded in the absence of S-9 in Figure 3.4.

Nytro-10GBN however, showed a further increase of 133 revertants when 80% v/v S-9 was used instead of 10% v/v (a total of 1137 revertants). When compared to revertant number without S-9 (837) it was further evidence that Nytro-10GBN contained indirect mutagens that might be PAHs. However, the presence of indirect mutagenicity was once again only observed at the highest dose. As carrier solvent saturation did not allow testing at higher doses, the results were validated by comparing the Grimmer extract mutagenicity data (at 500 mg plate^{-1}) with the mutagenicity data of other extraction methods (Section 3.6, 5.2 and 5.5) and indirect mutagenicity was concluded.

The number of revertants for oil 8 increased from 1228 without S-9 (Figure 3.6) to 2444 with 80% v/v S-9 (Figure 3.8). This near-doubling in number on the addition of S-9 was deemed significant, particularly as it was an increase of over 1000 revertants. Since oil 8 was observed to be 3 times richer in aromatics than oil 4 or Nytro-10GBN (IP 346 % w/w data), it was possible that this increase in mutagenicity was due to PAHs. However this increase was only observed at loadings of 500 mg plate^{-1}; all responses observed at 50 mg plate^{-1} or less were no greater than the response observed in the absence of S-9. Once again, issues of saturated carrier solvent meant that these results were validated using the mutagenicity data of alternative extracts (Section 3.6, 5.2 and 5.5) and only then was indirect mutagenicity concluded. Results are further discussed in Section 7.2.4 and compared to other extracts in Section 7.4.1 and 7.4.2.1.

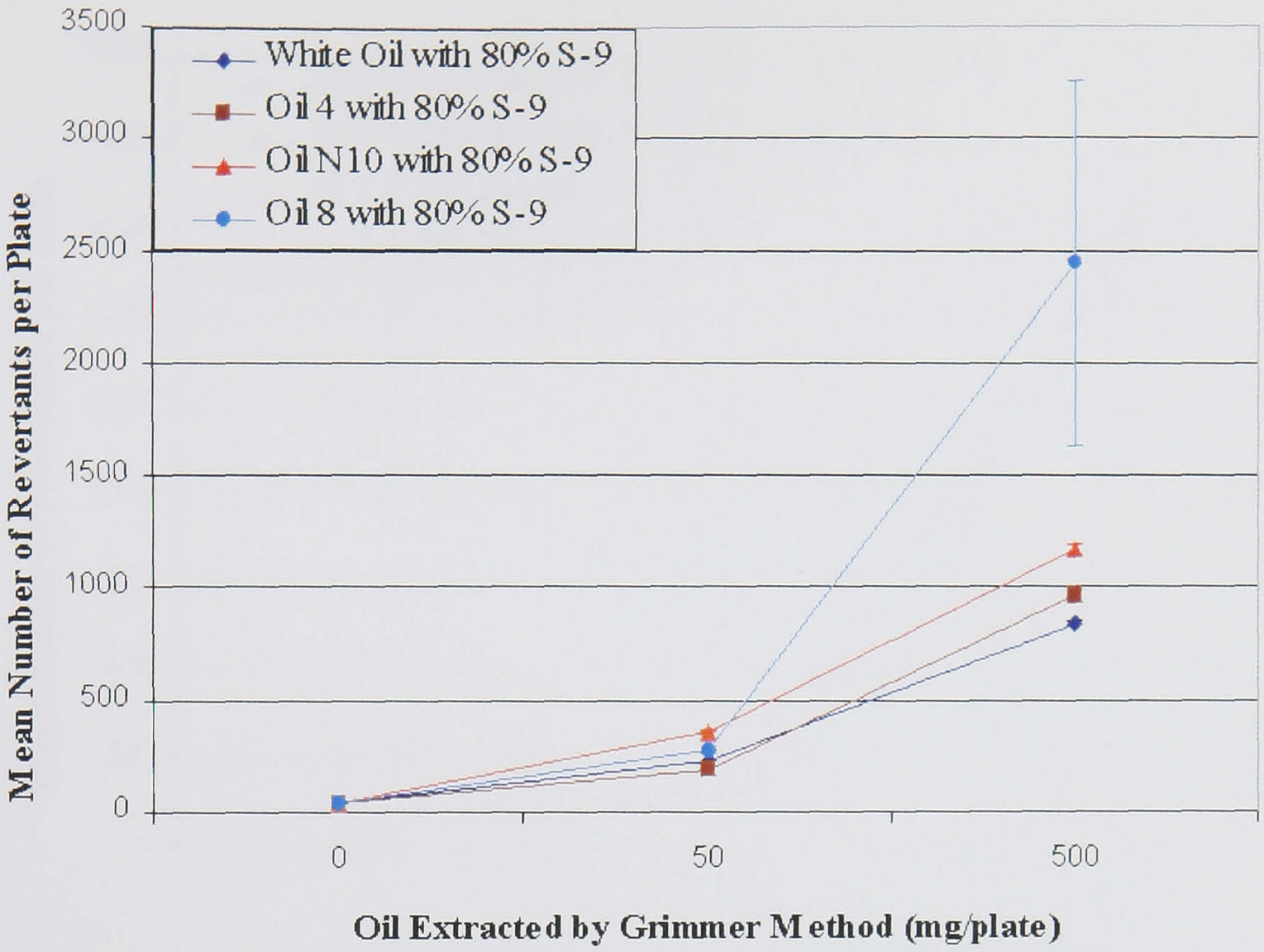


FIGURE 3.8. The Ames test revertants on the addition of Grimmer oil extracts with 80% v/v S-9 (n = 3). %CV ranges from 3-33%.

3.5 AMES TESTING OF AGED OIL GRIMMER EXTRACTS

In addition to new transformer oils, aged oils (Section 2.1.1) were extracted and tested to give an indication of how mutagenicity changed with age. This would indicate if mutagenic threat increased over the lifetime of the oil. The oil used was tested before ageing (ALT 0) and then tested after ageing for 1 week (ALT 1), 2 weeks (ALT 2) and 3 weeks (ALT 3). Results showed that the Grimmer extracts of all oils were mutagenic with or without 10% v/v S-9 (Figures 3.9, 3.10).

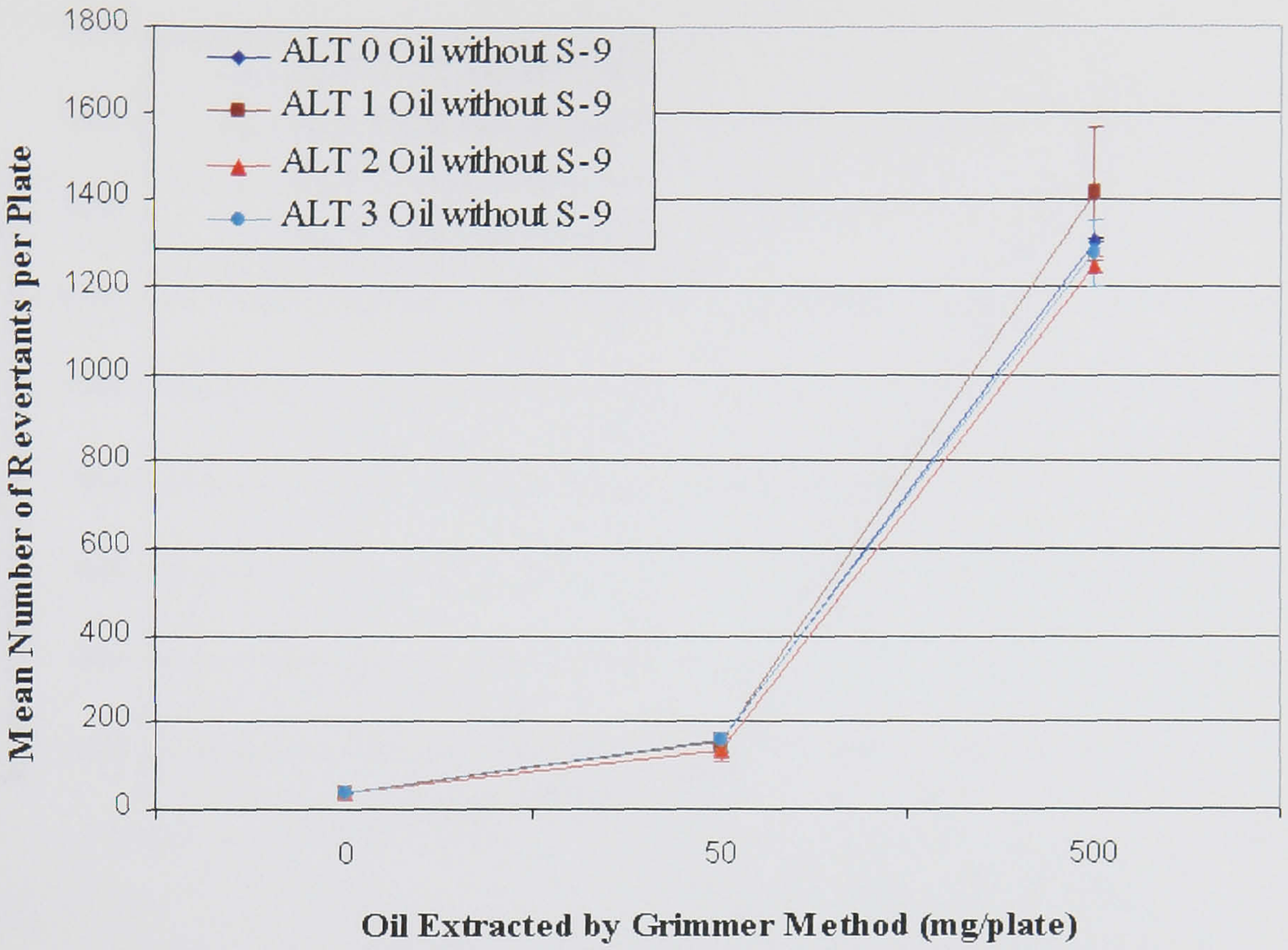


FIGURE 3.9. The Ames test revertants on the addition of Grimmer extracts of new oil ALT 0 and after ageing for 1 week (ALT 1), 2 weeks (ALT 2) and 3 weeks (ALT 3) without S-9 (n = 3). %CV ranges from 1.4-20.7%.

No significant change in mutagenicity was observed with artificial ageing of up to 3 weeks. There was an increase in the number of revertants by >200 on the addition of 10% v/v S-9 for the Grimmer extracts, although this did not constitute a doubling in revertant number compared to that observed without S-9. However, it was possible that the extra revertants were due to the presence of an indirect mutagen such as PAHs (Section 7.2.5). Further measures of aged oil mutagenicity were performed in Section 5.6 with alternative extracts and concluded that direct and indirect mutagenicity was present, but did not change with age.

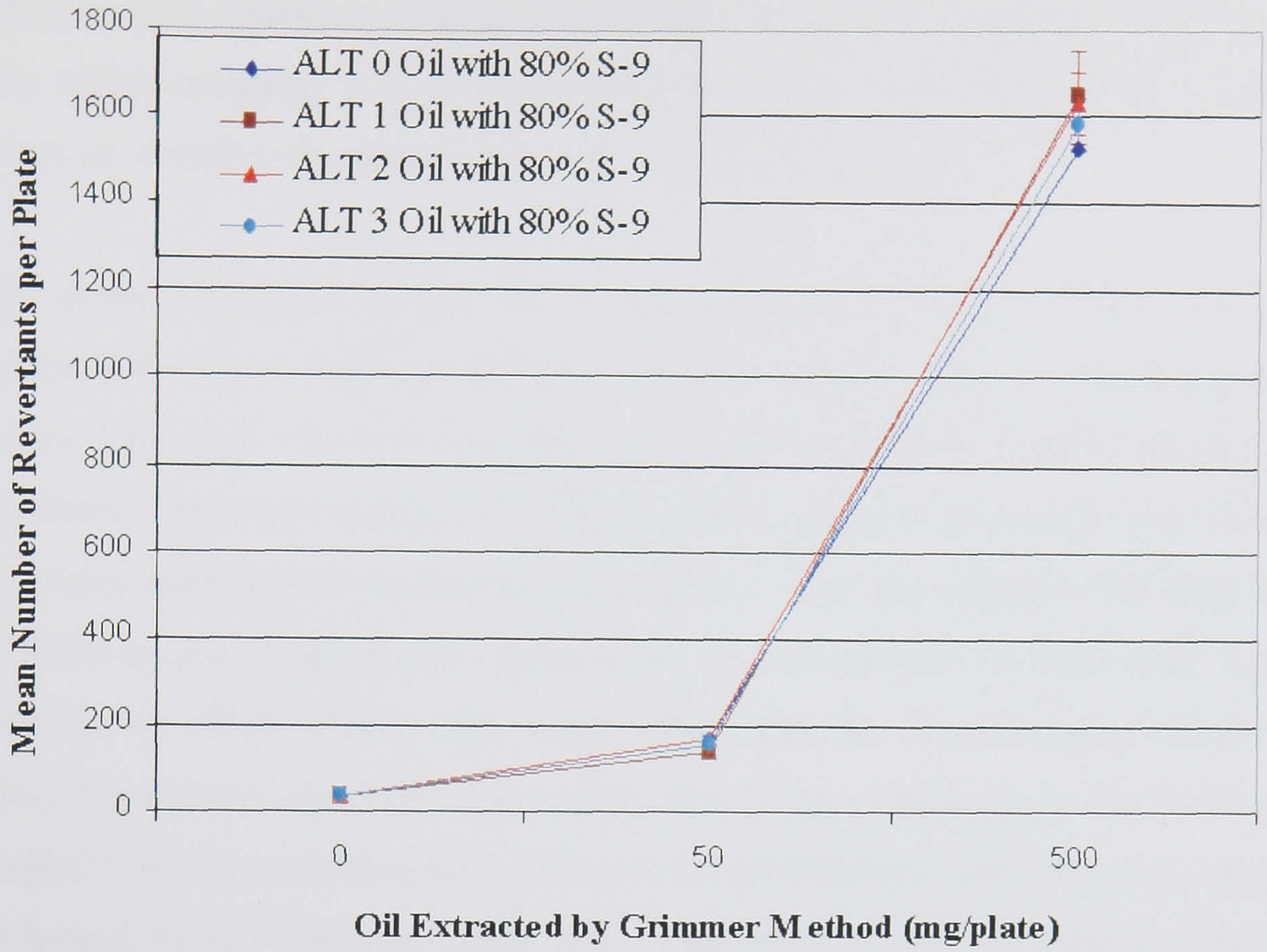


FIGURE 3.10. The Ames test revertants on the addition of Grimmer extracts of new oil ALT 0 and after ageing for 1 week (ALT 1), 2 weeks (ALT 2) and 3 weeks (ALT 3) with 80% v/v S-9 (n = 3). %CV ranges from 3.7-13.8%.

3.6 AMES TESTING OF FRACTIONATED SOLID PHASE EXTRACTS

A number of issues were encountered when testing liquid-liquid extracted oils with the Ames test. The first issue was that the IP 346 and Grimmer methods gave different results. In order to prevent false negative diagnosis, it was considered appropriate to use the most mutagenic result, and so the Grimmer extraction method was considered more appropriate than the IP 346 method.

In addition to this, mutagenicity testing proved to be expensive due to the requirement for 80% v/v S-9, an important consideration when large numbers of samples require

processing by the end-user. The next stage therefore, was to find a different method of extraction that correlates with the results of one of the extraction methods already used, whilst reducing S-9. It was postulated that by fractionating the aromatic oil extract, an overall reduction in S-9 usage was possible.

Solid phase extraction (SPE) was investigated as an alternative to LLE. It was postulated that this extraction method would also remove more unwanted components than the LLE methods, therefore allowing improved identification of PAHs in oil. It also allowed easy fractionation of PAHs according to their interaction with the sorbent so that any synergism or antagonism arising from both the presence of a large mixture of PAHs in one fraction and competitive or non-competitive inhibition would be overcome. A silica column was used, which was the recommended normal phase sorbent for moderately polar components. Due to the preference of the National Grid Company Plc for non-hazardous solvents, an investigation into the most appropriate low hazardous solvents was undertaken. The literature showed ethyl acetate to be a suitable elution solvent, so it was selected over dichloromethane, the preferred solvent for PAH analysis (Thurman and Mills, 1998).

3.6.1 Optimisation of Oil Volume for Silica Column Extraction

An experiment was performed to determine the most appropriate oil volume for silica (SiO_2) SPE efficiency. The more oil that could be extracted at once, the less time consuming the extraction. Consequently loadings of 10 mL and 20 mL were examined. Efficiency was determined by spiking the oil with the EPA 16 priority PAHs before extraction (Table 3.8). It was found that overall efficiency was improved when more oil (20 mL) was added. This may be due to loss of PAHs within the column with lower oil loadings, particularly as lower loadings required greater volumes of elution solvent. This experiment also gave an indication of efficiency for this large-scale extraction. It proved difficult to obtain quantitative data due to the presence of interferents illustrated by the high %CV values, and efficiency was found to vary widely particularly with respect to the smaller, less polar PAHs. For 20 mL of oil, 14 of the 16

PAHs were recovered while only 8/16 were recovered with 10 mL of oil. Extraction efficiencies ranged from 24-109% of 20 mL of oil, while 10 mL of oil led to an efficiency range of 28-87%.

TABLE 3.8. Extraction efficiencies of large scale solid phase extraction with 20 mL and 10 mL of Nytro-10GBN oil (n = 3).

Name of PAH	*20 mL Oil Nytro-10GBN: Efficiency of Extraction (%)*	*%CV for 20 mL Extraction*	*10 mL Oil Nytro-10GBN: Efficiency of Extraction (%)*	*%CV for 10 mL Extraction*
Naphthalene	24	35	0	0
Acenaphthylene	55	17	0	0
Acenaphthene	91	14	0	0
Fluorene	65	0	58	20
Phenanthrene	27	39	30	23
Anthracene	42	30	28	19
Fluoranthene	30	18	0.0	0
Pyrene	62	5	87	43
Benzo(a)anthracene	0	0	55	16
Chrysene	0	0	0	0
Benzo(b)fluoranthene	96	3	0	0
Benzo(k)fluoranthene	89	10	0	0
Benzo(a)pyrene	89	33	0	0
Indeno(1,2,3-c,d)pyrene	99	44	75.	13
Dibenz(a,h)anthracene	102	44	86	1
Benzo(g,h,i)perylene	109	31	78	18

3.6.2 Mutagenicity of Solid Phase Extracts

The SPE extracts were eluted in two fractions (Section 2.2.4.2) and each fraction was tested separately for mutagenicity with 10% v/v S-9. Oil 4 fraction 1 showed direct mutagenicity with the number of revertants increasing on addition of S-9 (increase of

200 revertants; 2.5 fold). Such mutagenicity had not been observed for the Grimmer extract. Fraction 2 proved non-mutagenic with or without S-9 (Figure 3.11). Nytro-10GBN also only showed mutagenicity in fraction 1 with and without S-9. In this case the number of revertants increased by just over 100 on the addition of S-9, an increase of 75% and therefore not a doubling (Figure 3.12).

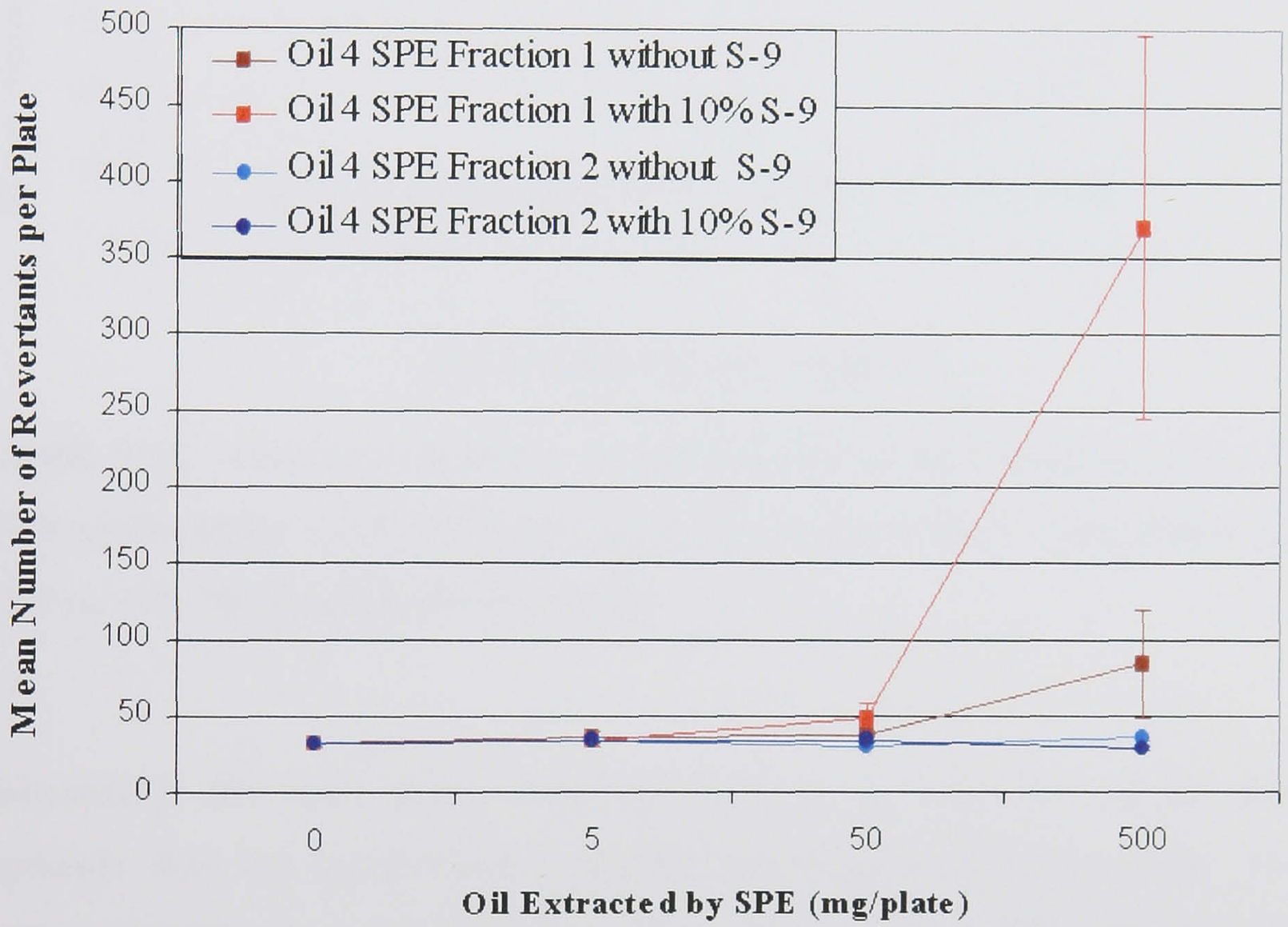


FIGURE 3.11. Ames test revertants on the addition of two fractions of oil 4 large produced by scale solid phase extraction in the absence and presence of 10% v/v S-9 (n = 9). %CV ranges from 1.4-42%.

Direct and indirect mutagenicity for fraction 2 of oil 8 was obtained, but fraction 1 with S-9 produced further indirect mutagenicity (Figure 3.13). The solid phase extraction results suggested that oil 8 contained additional mutagenic PAHs since indirect mutagenicity was observed in both fractions. This correlated with Grimmer results and IP346 %/ w/w values again, suggesting that oil 8 contained a greater PAHs loading than the other oils. This is further discussed in Section 7.2.6.

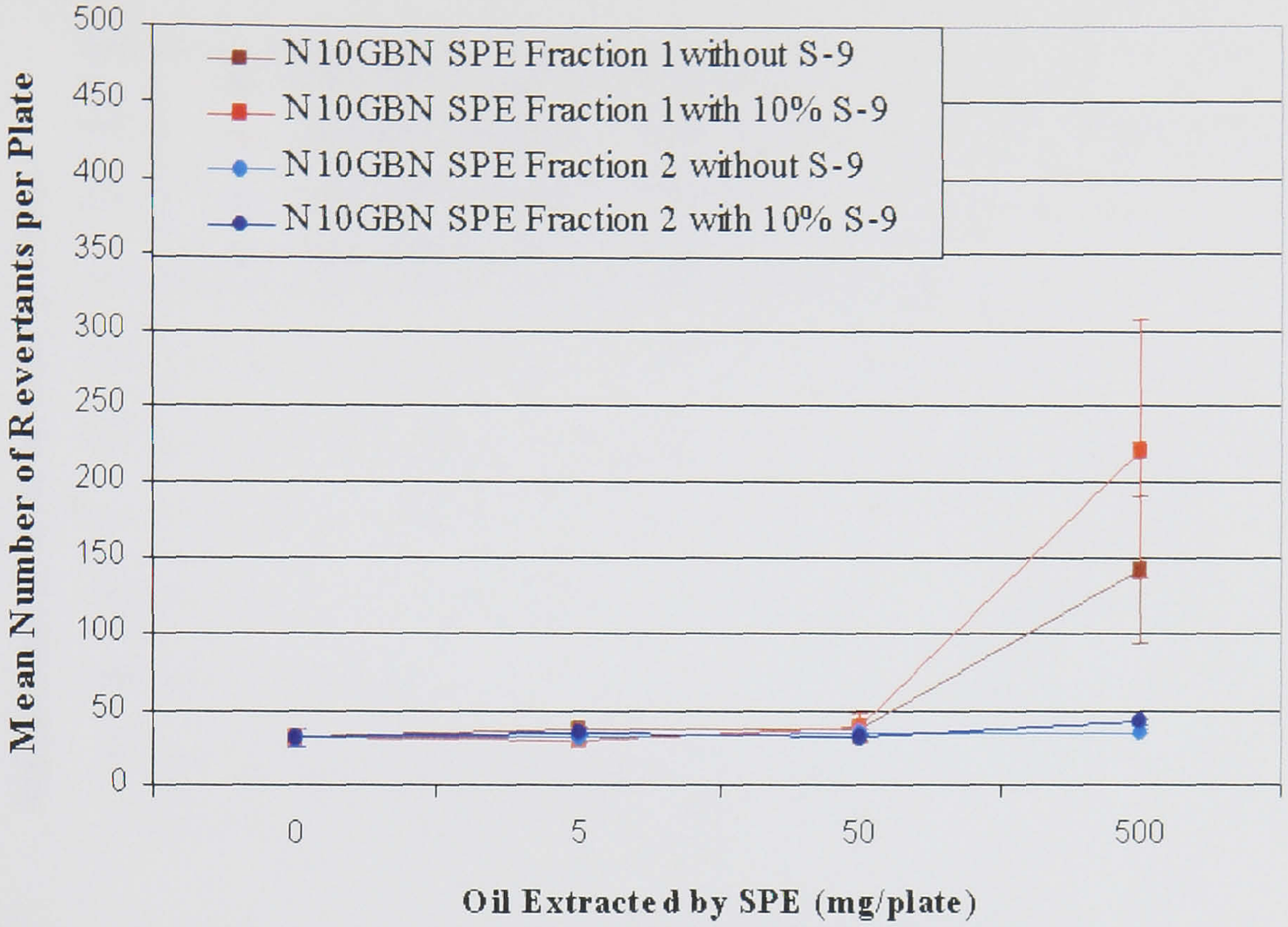


FIGURE 3.12. Ames test revertants on the addition of two fractions of oil Nytro-10GBN produced by scale solid phase extraction in the absence and presence of 10% v/v S-9 (n = 9). %CV ranges from 1.8-38%.

Unfortunately, the solid phase extraction proved less able to remove aliphatic compounds than the liquid-liquid extraction method due to immiscibilty with the aqueous environment of the Ames test plates. This inability to mix on the plate was believed to be the cause of large variations in the Ames test results and makes them less conclusive that the Grimmer extract results. Definite trends only became apparent when the test was repeated on at least nine plates. Figure 3.14 shows the TIC of the two fractions of Nytro-10GBN. Again, the complexity of the chromatograms did not allow individual PAH species or other possible mutagens to be identified.

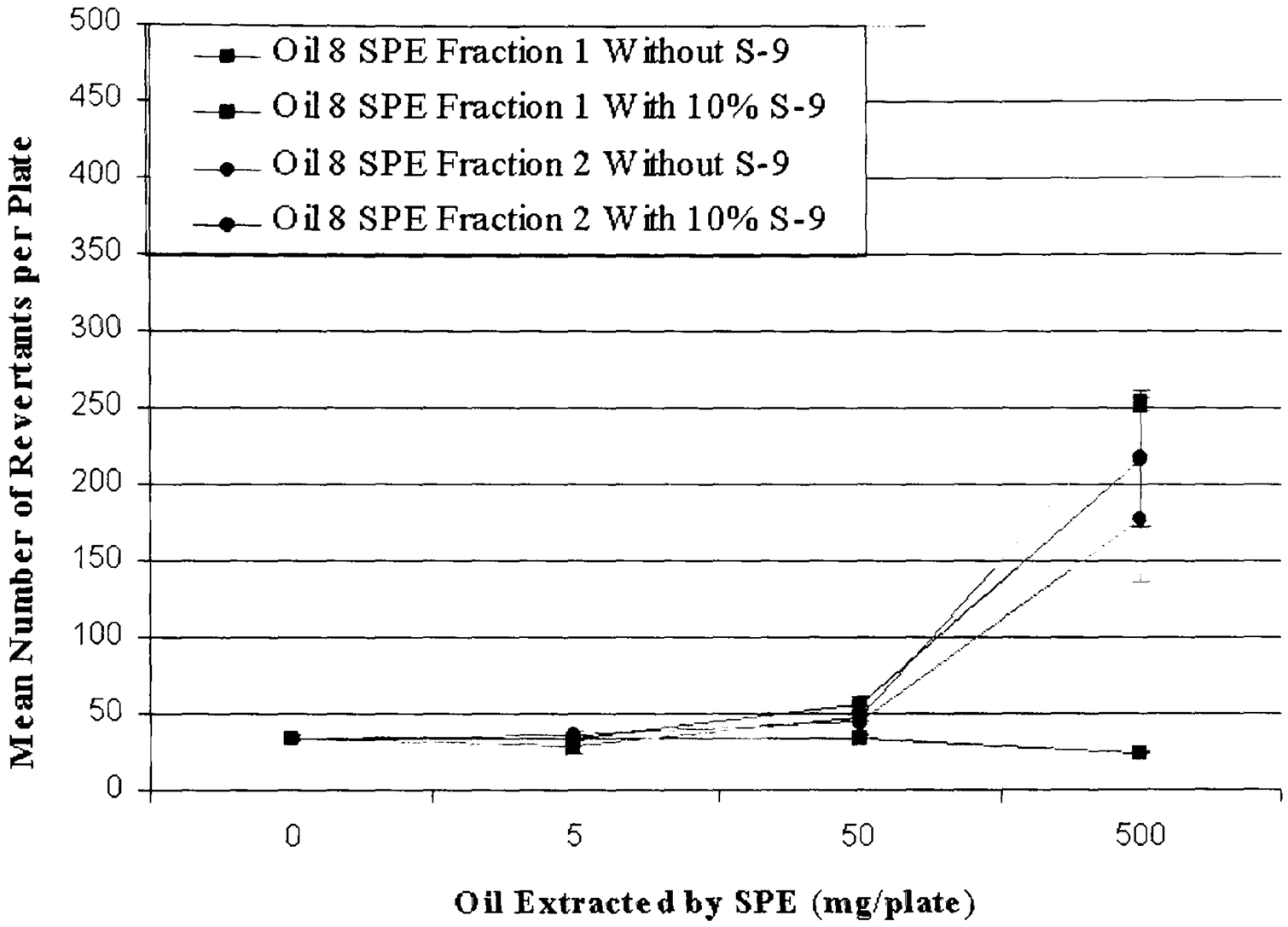


FIGURE 3.13. Ames test revertants on the addition of two fractions of oil 8 produced by scale solid phase extraction in the absence and presence of 10% v/v S-9 (n = 9). %CV ranges from 2.7-25%.

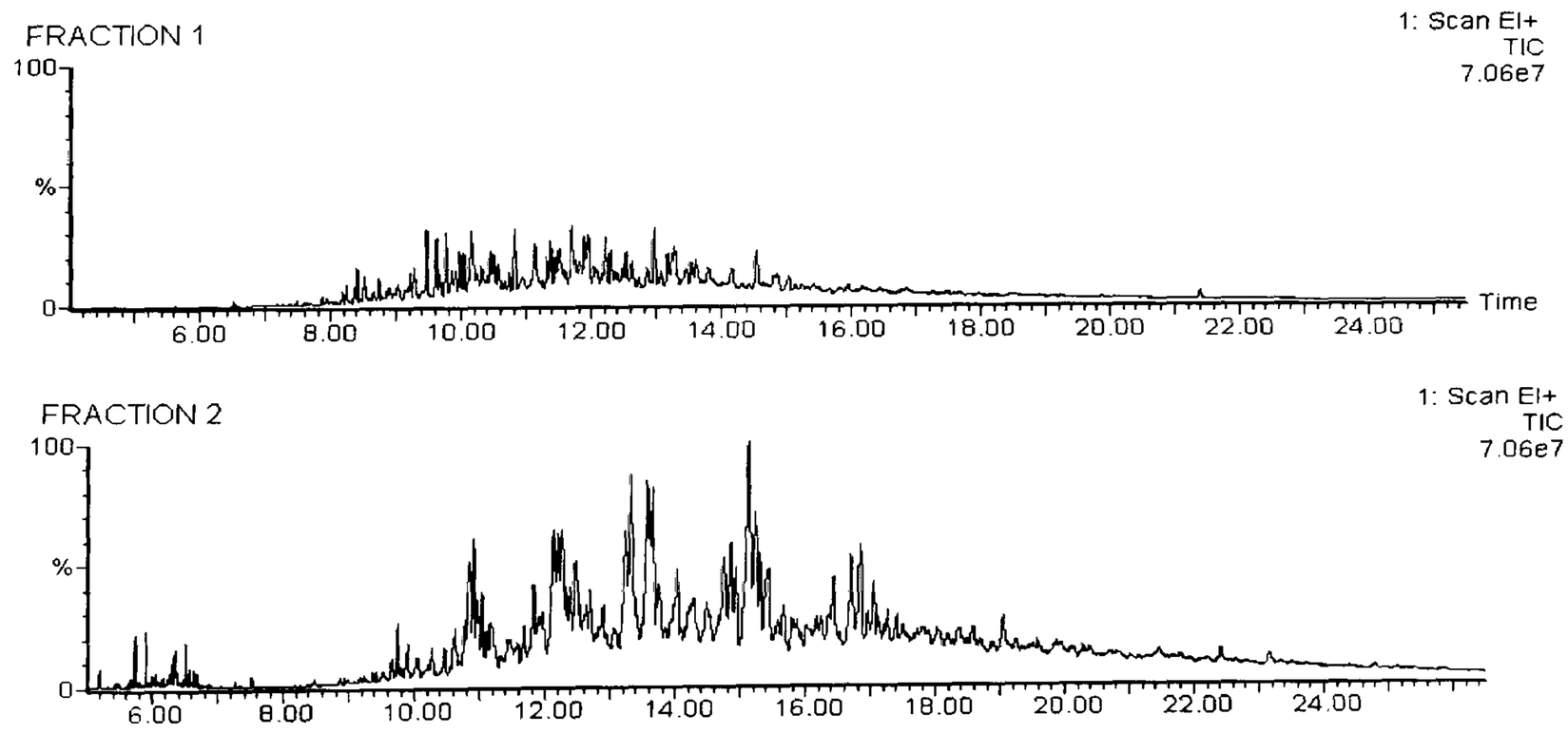


FIGURE 3.14. Total ion chromatogram of the two PAH fractions of Nytro-10GBN from silica solid phase extraction.

However, the results of the solid phase extraction tests have shown that most mutagens could be confined to one fraction. This had potential for concentrating the extract and removing interferents. In addition, indirect mutagens had been observed in oil 4, that had not been observed in the Grimmer extract. Nevertheless, the liquid-liquid extraction method did have benefits over the SPE method, in that the former method was better at removing aliphatics and was therefore more repeatable. These findings suggested that the oil preparation method would benefit from a combination of liquid-liquid extraction to remove the aliphatics, followed by solid phase extraction to fractionate and isolate the bulk of the mutagenic species.

3.7 AMES TESTING OF GRIMMER/SPE EXTRACTS

The Grimmer method was used in conjunction with a small scale solid phase extraction to generate two extract fractions and to evaluate if this double extraction method gave a cleaner extract than a single extraction method. The Grimmer method LLE was used rather than the Blackburn method, as cyclohexane was used as the final solvent and hence was compatible with SPE. The final solvent in the Blackburn method was DMSO, which was difficult to evaporate without possible analyte losses due to low volatility. The results are shown in Figure 3.15. Unlike the Grimmer or SPE methods individually, no mutagenicity or toxicity was observed for any of the oil extracts even with fractionation at 10% v/v S-9.

The TIC scan in Figure 3.16 shows than the extracts were still very complex and no information on composition could be obtained. Due to the lack of mutagenicity of the extracts, the wash material from the solid phase column was tested for mutagenicity. The results, also shown on Figure 3.15 indicated no mutagenicity or toxicity. It was not fully understood why the extracts no longer exhibited mutagenicity. It was possible that a proportion of the PAHs were retained on the silica column or were distributed between the cyclohexane wash and the two aromatic fractions so that no single fraction contained sufficient PAHs to cause a mutagenic response. In conclusion, as the TIC scan shows, this extraction method was no better than the single step methods for

cleaning and identifying PAHs, and so was not investigated further. For exploring mutagenicity, the liquid-liquid or single step solid phase extractions were superior, even though all extraction methods examined thus far did not allow the identification of the compounds causing mutagenicity.

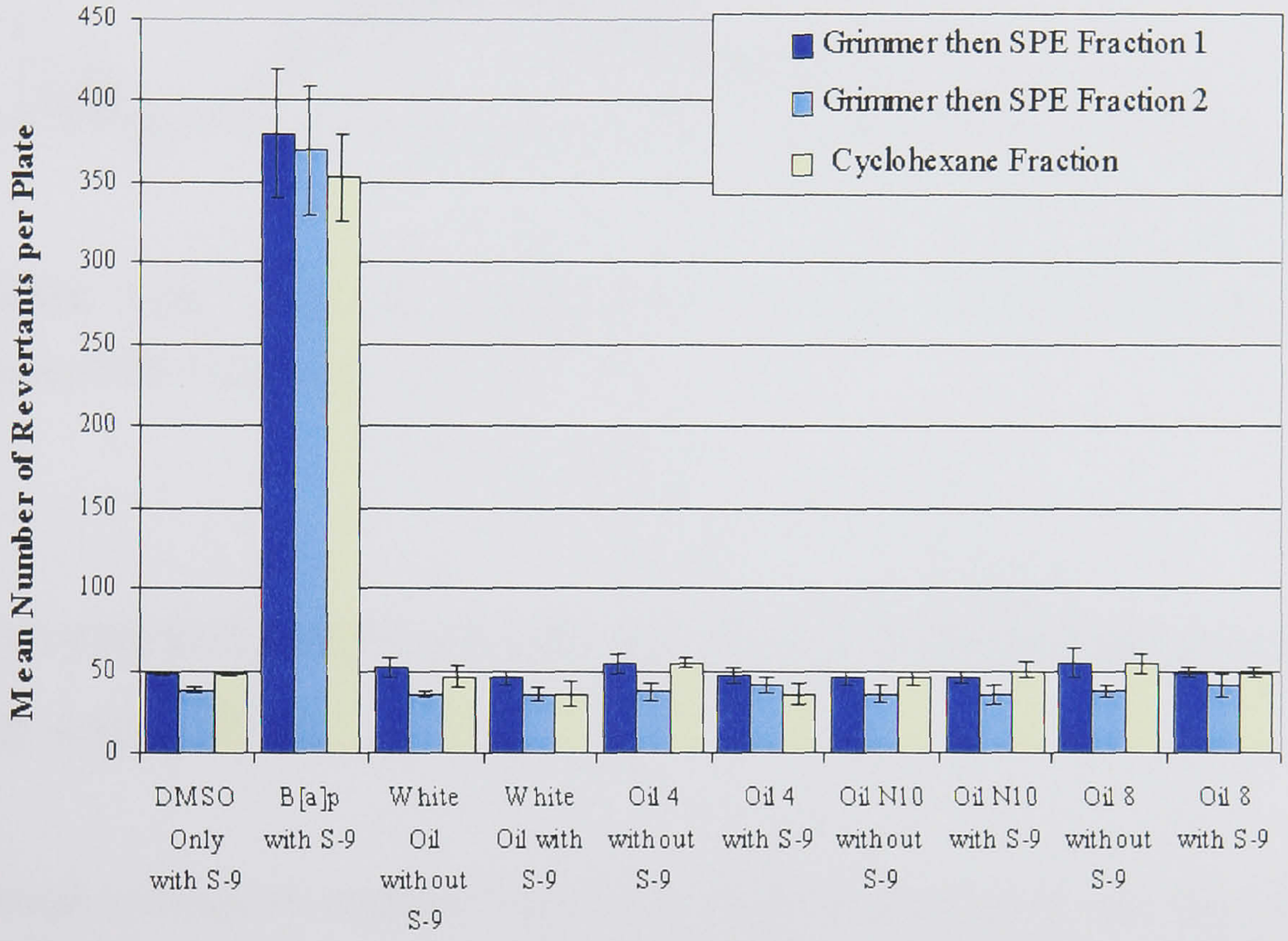


FIGURE 3.15. Ames test revertants on the addition of Nytro-10GBN Grimmer/SPE extracts in the absence and presence of 10% v/v S-9. Extracts are compared to the revertants on the negative (DMSO only) and positive (B[a]p with 10% v/v S-9) control plates.

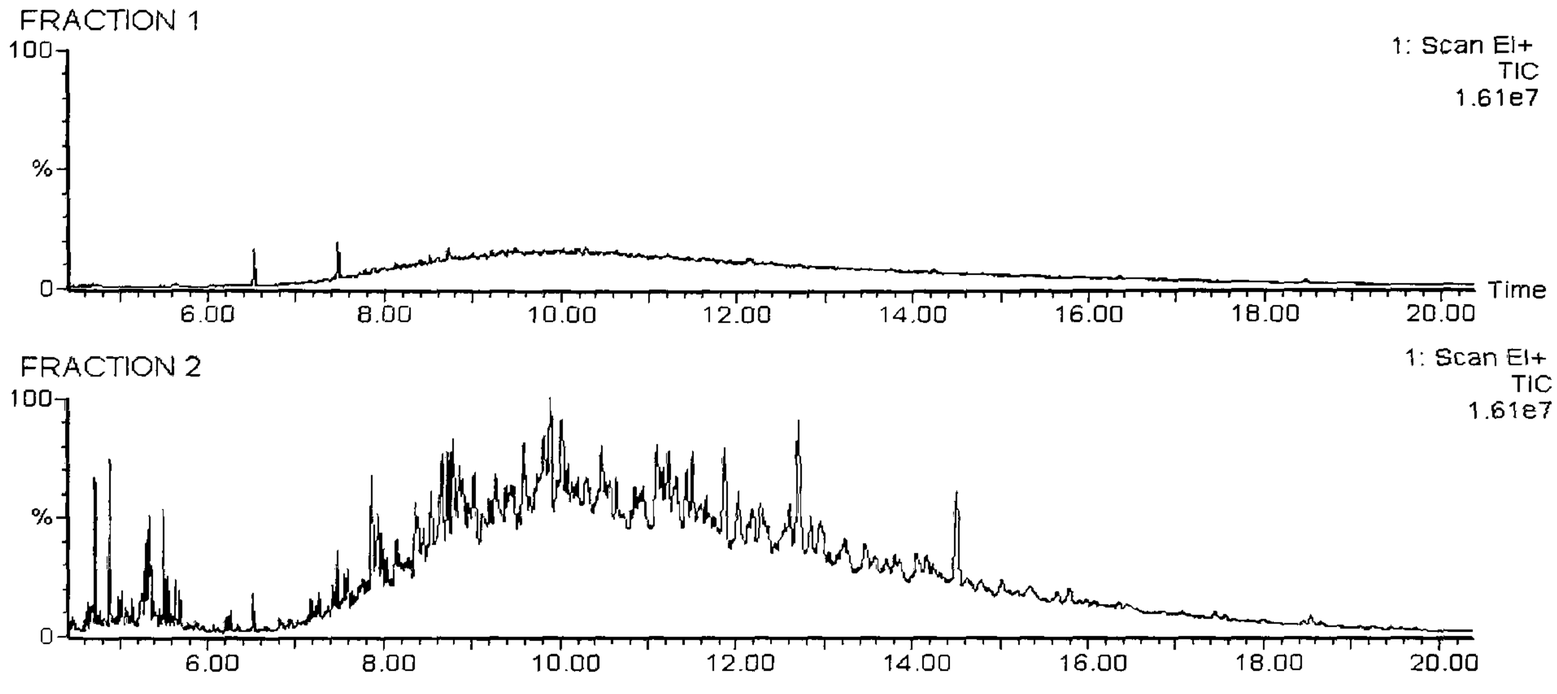


FIGURE 3.16. Total ion chromatogram of Nytro-10GBN fractionated by the Grimmer/SPE extraction.

3.8 INVESTIGATION OF OIL EXTRACT INTERFERENCE ON THE AMES TEST

Although a mutagenic response had thus far been observed for oil extracts, due to their complex nature, it was postulated that extracting the aromatic fraction had not removed all antagonistic or synergetic effects. It particular, identifying antagonistic affects on PAH mutagenicity was of interest as it was possible that the full extent of PAH mutagenicity had not yet been observed. The inhibition of benzo[a]pyrene and 2-amino anthracene mutagenicity had previously been detected when in the presence of whole oil (Section 3.3.3). It was postulated that these inhibitors of PAH mutagenicity may have been extracted with the aromatic fraction of the oil.

To determine if the oil extracts would alter the mutagenicity of PAHs, 50 mg plate^{-1} of the extracts were added to the bacteria in turn, with benzo[a]pyrene (50 μg mL^{-1}) or 2-amino anthracene (20 μg mL^{-1}). This amount of oil extract was used as it was the highest dose of extract tested that did not produce a mutagenic response, with or without S-9. In this way, only the mutagenicity of the benzo[a]pyrene/2-amino

anthracene would be observed as it would be difficult to subtract benzo[a]pyrene/2-amino anthracene mutagenicity from oil extract mutagenicity, when oil extract mutagenicity can have a large standard deviation. Only 10% v/v S-9 was used at this stage, as benzo[a]pyrene in the control plates had only required 10% v/v to be activated.

The results in Table 3.9 shows the reduction in benzo[a]pyrene or 2-amino anthracene mutagenicity when the Grimmer oil extracts were added. There was an increase in inhibition with increasing PAH content, with inhibition no greater than 32%. These trends were present with whole oils (Section 3.3.3). The inhibition was significant according to the t-test results in Table 3.9 as all extracts gave a value more negative than the critical value of –2.35 (n = 3). Such an increase in inhibition when a high PAH content oil was used was investigated further in Section 5.7 where S-9 was increased to see how it affected inhibition.

TABLE 3.9. Effects on revertant number of adding Grimmer extracts to mutagens (benzo[a]pyrene and 2-amino anthracene).

Sample on Plate	*Mean Number of revertants*	*± SD (n = 3)*	*%CV*	*Reduction in Revertants from Mutagen on Exposure to Oil (%)*	*One-Sided T-Test (t)**
Benzo[a]pyrene (B[a]P0	196	34	17	0	N/A
Grimmer extract of White oil and B[a]P	165	9	5	15	-5.7
Grimmer extract of Oil 4 and B[a]P	157	14	9	20	-4.9
Grimmer extract of Oil 8 and B[a]P	134	8	6	32	-13.4
2-amino anthracene (2-aa)	729	37	5	0	N/A
Grimmer extract of White oil and 2-aa	638	44	7	12	-3.5
Grimmer extract of Oil 4 and 2-aa	568	94	16	22	-2.9
Grimmer extract of Oil 8 and 2-aa	498	64	13	32	-6.2

* *Critical Value t_3 = -2.35 (P = 0.05)*

SPE extracts in Table 3.10 again followed the trend, as all oils caused significant inhibition according to the t-test values with the greatest inhibition from oil 8 extracts (30%) as did the Grimmer/SPE extracts, although the inhibition was up to 43% for oil 8 (Table 3.11).

TABLE 3.10. Effects on revertant number of adding solid phase extraction fractions 1 and 2 to mutagens (benzo[a]pyrene and 2-amino anthracene).

Sample on Plate	*Mean Number of revertants*	*± SD (n = 3)*	*%CV*	*Reduction in Revertants from Mutagen on Exposure to Oil (%)*	*One-Sided T-Test (t) **
Benzo[a]pyrene (B[a]P)	196	34	17	0	N/A
SPE Fraction 1 White oil and B[a]P	165	17	10	16	-3.1
SPE Fraction 1 Oil 4 and B[a]P	159	10	6	19	-6.7
SPE Fraction 1 Oil 8 and B[a]P	146	19	13	26	-4.5
2-amino anthracene (2-aa)	729	37	5	0	N/A
SPE Fraction 1 White oil and 2-aa	622	77	12	15	-6.8
SPE Fraction 1 Oil 4 and 2-aa	564	64	11	23	-8.6
SPE Fraction 1 Oil 8 and 2-aa	614	16	3	16	-8.0
Benzo[a]pyrene (B[a]P)	196	34	17	0	N/A
SPE Fraction 2 White oil and B[a]P	174	6	3	11	-2.4
SPE Fraction 2 Oil 4 and B[a]P	153	9	6	22	-4.4
Grimmer the SPE Fraction 2 Oil 8 and B[a]P	138	12	9	30	-12.5
2-amino anthracene (2-aa)	729	37	5	0	N/A
SPE Fraction 2 White oil and 2-aa	634	22	3	13	-7.4
SPE Fraction 2 Oil 4 and 2-aa	612	53	9	16	-3.8
SPE Fraction 2 Oil 8 and 2-aa	536	53	10	26	-6.2

* *Critical Value t_3 = -2.35 (P = 0.05)*

TABLE 3.11. Effects on revertant number of adding Grimmer/solid phase extraction fractions 1 and 2 to mutagens (benzo[a]pyrene and 2-amino anthracene).

Sample on Plate	*Mean Number of revertants*	*± SD (n = 3)*	*%CV*	*Reduction in Revertants from Mutagen on Exposure to Oil (%)*	*One-Sided T-Test (t) **
Benzo[a]pyrene (B[a]P)	196	34	17	0	N/A
Grimmer/SPE Fraction 1 White oil and B[a]P	165	17	10	16	-3.1
Grimmer/SPE Fraction 1 Oil 4 and B[a]P	159	10	6	19	-6.7
Grimmer/SPE Fraction 1 Oil 8 and B[a]P	132	4	3	32	-26.3
2-amino anthracene (2-aa)	729	37	5	0	N/A
Grimmer/SPE Fraction 1 White oil and 2-aa	555	72	13	24	-9.1
Grimmer/SPE Fraction 1 Oil 4 and 2-aa	557	68	12	24	-8.6
Grimmer/SPE Fraction 1 Oil 8 and 2-aa	415	18	4	43	-7.7
Benzo[a]pyrene (B[a]P)	196	34	17	0	N/A
Grimmer/SPE Fraction 2 White oil and B[a]P	160	7	4	18	-4.1
Grimmer/SPE Fraction 2 Oil 4 and B[a]P	153	9	5	22	-4.3
Grimmer/SPE Fraction 2 Oil 8 and B[a]P	131	14	11	33	-30.9
2-amino anthracene (2-aa)	729	37	5	0	N/A
Grimmer/SPE Fraction 2 White oil and 2-aa	568	63	11	22	-4.4
Grimmer/SPE Fraction 2 Oil 4 and 2-aa	512	53	10	30	-7.1
Grimmer/SPE Fraction 2 Oil 8 and 2-aa	432	42	10	41	-12.3

** Critical Value t_3 = -2.35 (P = 0.05)*

In order for inhibition to change an Ames test result, it would have to reduce mutagenicity by 50% (therefore disguising a doubling in revertant number). Inhibition of up to ~30-40% observed here would only be significant if the Ames test had produced an intermediate (inconclusive) result, as in the case of chrysene (Section 3.2.1.1). As no result thus far gained for oil extracts with the Ames test was inconclusive, the inhibition measured here had not influenced the Ames test results. These findings suggested however, that any oil extracted in the future, that produced a

weakly positive or intermediate result, should be tested for inhibition from other components in the mixture.

3.9 CONCLUSIONS

In this chapter mutagenicity has been found with and without S-9 with two different extraction methods (Grimmer and SPE). The Ames test indicated the presence of direct mutagens suggesting that mutagens other than PAHs are present in the oil. The Grimmer extracts in particular, indicated that mutagenicity increased when S-9 was added for oils that contained intermediate (Nytro-10GBN) or high levels (oil 8) of PAH according to IP 346 % w/w data. This suggested that PAHs could be causing additional mutagenicity in the oil. However, due to the complexity of the GC-MS data, it was not possible to specifically identify PAHs, and therefore it cannot be concluded that the indirect mutagen observed was a PAH. Without identifying the specific contribution of PAHs to oil mutagenicity, the benefits or costs of having PAHs in the oil cannot be assessed. For this reason a new method of extraction is required. Preferably the method will generate an extract that can be used in the Ames test, but is clean enough to analyse with GC-MS so that mutagenic effect can be directly correlated to identified components. Development of such an extraction method is detailed in chapter 4.

CHAPTER 4.0

RESULTS

DEVELOPMENT OF A POLYCYCLIC AROMATIC HYDROCARBON EXTRACTION METHOD FOR TRANSFORMER OILS USING GC-MS ANALYSIS

4.1 INTRODUCTION

As seen in Chapter 3, methods for oil extraction using liquid-liquid extraction (LLE) methods at 80% S-9 and silica solid phase extraction (SPE) using 10% S-9 were successful at isolating mutagenic components in the oil. Direct mutagenicity was observed from a non-polyaromatic source, while an increase in mutagenicity on the addition of activation enzymes was attributed to the presence of PAHs in oil Nytro-10GBN and oil 8, as PAHs are indirect mutagens. However, it was not possible to identify PAHs or other mutagens in the oils by GC-MS as the extracts were still complex mixtures of unresolved peaks.

Therefore, a new extraction method was required, that was more specific to PAHs, especially those considered carcinogenic. Such an extraction method would allow identification of potential carcinogenic PAHs without the requirement for mutagenicity testing, and would ensure that any mutagenicity observed with the Ames test could be directly linked to oil PAH content in the oil. Currently there is no extraction method that can be used with an analytical technique, such as GC-MS whilst providing a sufficient extract to test for mutagenicity. It was therefore the aim of this work to find a preparative extraction method that provided an extract of sufficient quantity for Ames testing, with sufficient purity for meaningful GC-MS analysis.

4.1.2 Solid Phase Extraction Methods for PAH Analysis

The LLE methods examined in Chapter 3 were crude methods that extracted the aromatic components from the oil. It proved difficult to improve these extractions without the addition of further clean-up or purification steps. SPE was considered a promising method of further sample clean-up due to operational simplicity. Although LLE followed by SPE clean-up proved to be unsuccessful (Section 3.7) it was believed that improvements could be made with further development and the use of alternative sorbents. SPE is commonly used for the extraction of PAHs from soil and water matrices, so was an obvious choice for purification of the sample. For these reasons

SPE methods were explored, primarily to identify improved sorbent-sorbent systems prior to use in combination with LLE or other SPE methods.

4.1.2.1 Alternative Solvents

The EPA had many standard extraction methods for extracting PAHs from a wide variety of matrices (EPA, 2002), but all involved hazardous solvents such a dichloromethane or hexane, which the National Grid Company Plc wished to avoid. For this reason, no pre-existing extraction method was utilised but several existing methods were used as the initial starting point for method development.

4.1.2.2 Column Size and Oil Volume

Sample loading and column dimensions are known to have an impact on the efficiency of the extraction method so both were considered carefully. The results from the large scale silica extraction method (Section 3.6) suggested that the extraction efficiency cannot be established without significant practical application. This was particularly true for complex mixtures such as oils.

Column dimensions were determined by commercial availability. Two sizes of column were used, a 15 g sorbent column and a 1.2 g sorbent column. When silica (SiO_2) and alumina (Al_2O_3) were directly compared for extraction efficiency, the 15 g column was preferred (Section 4.2 and 4.3.1). This was because larger quantities of spiked oil could be extracted and analysed therefore reducing the error associated with quantifying PAHs with GC-MS. This was particularly important when two sorbents were directly compared or when the extraction efficiency of the system was very low.

A series of experiments were performed using a number of sequential SPE extraction steps. These were performed on a small scale due to sorbent availability. Sorbents included cyanopropyl, Isolute PAH HC and C18, and were readily available in 1g cartridges. They were also useful for the initial screening of sorbents, as they required

less solvent and were less time consuming to perform. However, with less oil throughput, the error in quantifying poorly extracted PAHs is increased. This was remedied in a number of ways. The GC-MS sensitivity was repeatedly checked by running standards, the sample was concentrated to 1 mL before analysis and additional sample repeats were run (4-6 repeats rather than triplicate). Column loading could not be increased as this would saturate the column and affect extraction efficiency.

White oil was used for initial investigation of extraction methods as it contained little or no PAHs. Residual native PAHs would therefore not significantly interfere with spiking experiments. A 2 mL volume of an EPA 16 priority PAH standard mixture (10 $\mu g\ mL^{-1}$) was added to 10 mL of white oil and the solvent evaporated to dryness (2 $\mu g\ mL^{-1}$) at room temperature and used as required. White oil as a highly refined oil, is less complex than transformer oil, and was only used for selective ion chromatogram (SIC) quantification of the spike as its limitations were recognised (Section 2.1.1). Therefore extractions were performed with Nytro-10GBN in total ion chromatogram (TIC) mode to determine the extent of extraction of additional oil components in the less refined transformer oil.

4.2 DEVELOPMENT OF SILICA SOLID PHASE EXTRACTION

Initial work was performed to determine the volume of sample and solvent most effective for each silica column format. In cases where a single analyte requires extraction, significant method development can be achieved by reviewing analyte properties, such as structural information, functional group properties, solubility, pH, ionic strength etc. However, in this case, the complexity of the matrix and the mixture of unknown PAHs meant that the suitability of a SPE method had to be elucidated empirically rather than theoretically.

4.2.1 Amount of Oil Used for Extraction

Silica (SiO_2) was considered a good sorbent choice as it is commonly used for the separation of polar components from lipids, with alumina ($A_{l2}O_3$) often used as an alternative (Thurman and Mills, 1998). Various amounts of oil were passed down a 1.2 g (3 mL volume) silica column, to determine the maximum amount of oil that could be extracted without compromising the effectiveness of the washing step. The oil (Nytro-10GBN) was spiked with 1 μg mL^{-1} of each EPA 16 priority PAH. An UV lamp was used to follow the fluorescence arising from the aromatic fraction. GC-MS SIC was used to analyse the cyclohexane eluent to determine the point at which PAHs began eluting from the column. It was found that an oil loading of 50-100 μL onto a 1.2g column was optimum (data not shown). A ~1:10-20 oil: sorbent ratio was used as a guideline for further development purposes. This wide ranging ratio was used as the oils had a range of PAH loadings, which may affect extraction efficiency. It was also found that 5-6 mL of cyclohexane per gram of sorbent was suitable for washing purposes. This was found to be dependent on column dimensions such as plate number (Section 1.5.3.2), so modifications were made when appropriate.

4.2.2 Measuring Naturally Occurring PAHs in White "Blank" Oil with Silica Extraction

Silica and alumina SPE were used to extract PAHs from white oil to test its effectiveness as a blank oil in spiking experiments. Although neither sorbent was likely to be completely efficient (efficiency was tested in Section 4.3.1) an indication of PAH interference was established. Unspiked white oil was extracted with SiO_2 and Al_2O_3 sorbents through 15 g sorbent 10 mm x 46 cm columns (Section 2.2.4.1) and GC-MS SIC analysis with the Turbomass software was performed (Section 2.2.6.1) to determine the presence of the EPA 16 priority PAHs. Once the presence of native PAHs was established, this experiment was not repeated until a different sorbent showed enough potential to warrant further development.

TABLE 4.1. EPA 16 priority PAHs detected in white oil using GC-MS SIC quantification using Turbomass software.

Name	*Silica Extraction of 1 mL White oil (μg mL^{-1})*	*SD*	*Alumina Extraction of 1 mL White Oil (μg mL^{-1})*	*SD*
Naphthalene	0	0	0	0
Acenaphthylene	0	0	0	0
Acenaphthene	0	0	0	0
Fluorene	0.016	0.017	0.025	0.01
Phenanthrene	0.023	0.0012	0.013	0.011
Anthracene	0	0	0	0
Fluoranthene	0	0	0	0
Pyrene	0	0.011	0	0
Benzo(a)anthracene	0	0	0	0
Chrysene	0	0	0	0
Benzo(b)fluoranthene	0	0	0	0
Benzo(k)fluoranthene	0	0	0	0
Benzo(a)pyrene	0	0	0	0
Indeno(1,2,3-c,d)pyrene	0	0	0	0
Dibenz(a,h)anthracene	0	0	0	0
Benzo(g,h,i)perylene	0	0	0	0

Results in Table 4.1 show that no native PAHs were detected in white oil using SiO_2 or Al_2O_3 SPE except fluorene, phenanthrene and pyrene. However, all 3 PAHs were present at very low levels (<0.025 μg mL^{-1}) and as the oil was to be spiked at the 2μg mL^{-1} level the presence of these naturally occurring PAH was not considered significant.

4.2.2.1 Solvent Selection for Solid Phase Extraction

A range of solvents was examined with respect to the SPE method. Solvents were categorised according to their eluotropic strength (Table 1.3). Cyclohexane was the preferred solvent for washing, as it was less hazardous than hexane but had one of the lowest eluotropic strengths. The efficacy of three solvents (ethyl acetate, acetone and methanol) in eluting 1 mL of spiked white oil (2 μg mL^{-1}) from a 15 g silica column was examined (n = 3). Acetone and methanol in particular were identified as good solvents for the extraction of PAHs (Noordkamp *et al.*, 1997). Methanol was found to be unsuitable however, as the initial TIC showed significant column bleed (Figure 4.1). This was found to be from the silica extraction column rather than the GC column, as running pure methanol through the GC column did not have the same effect.

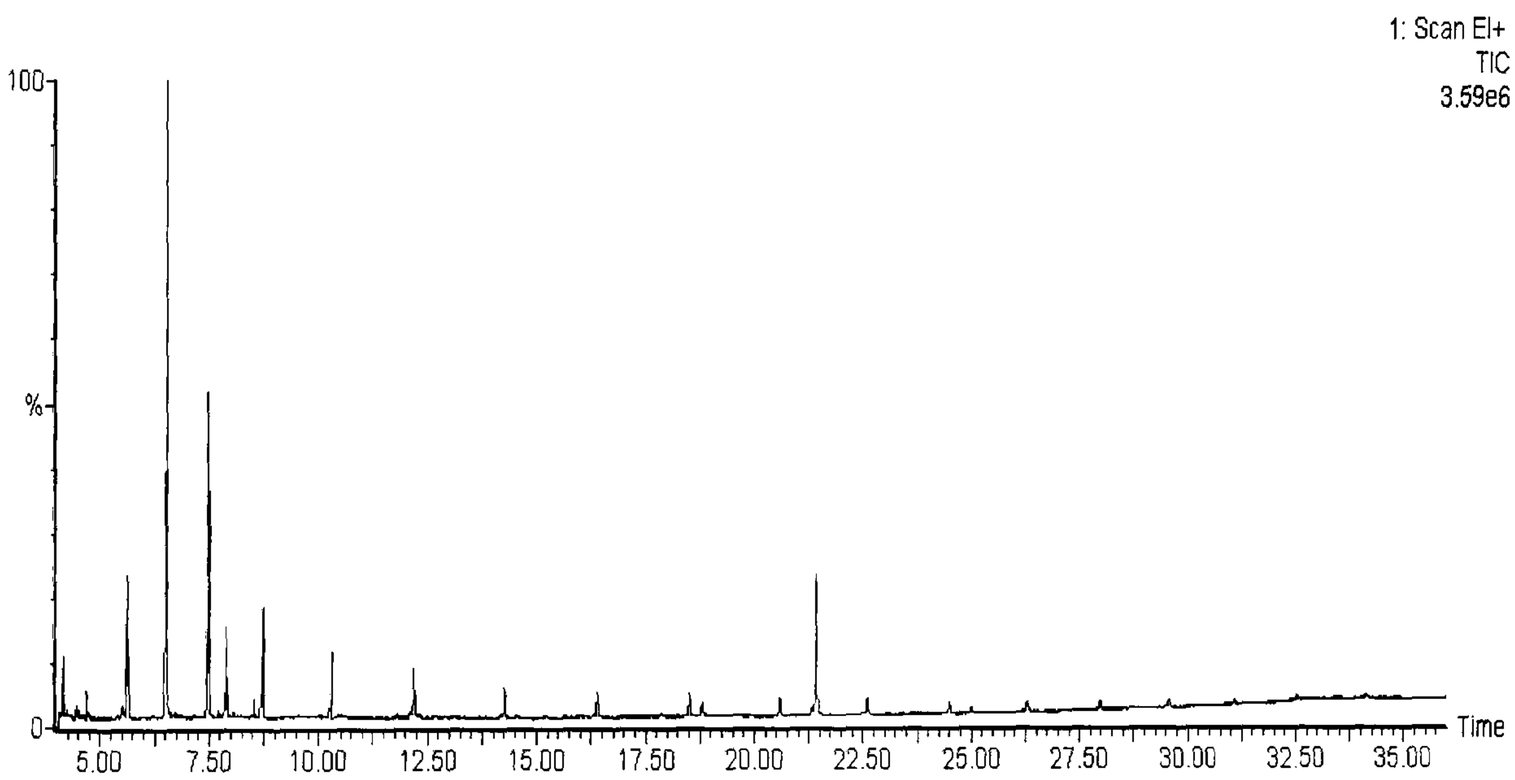


FIGURE 4.1. The TIC of Nytro-10GBN silica solid phase extraction with methanol elution. The peaks were identified as bleed from the silica extraction column.

Figure 4.2 shows a comparison of acetone and ethyl acetate extraction efficiency. Acetone proved the most efficient eluent for all EPA 16 priority PAHs except fluorene and fluoranthene where ethyl acetate was better by 0.06 and 0.03 μg mL^{-1} respectively.

However, as acetone was the most effective for carcinogenic PAHs, it was selected as the preferred solvent.

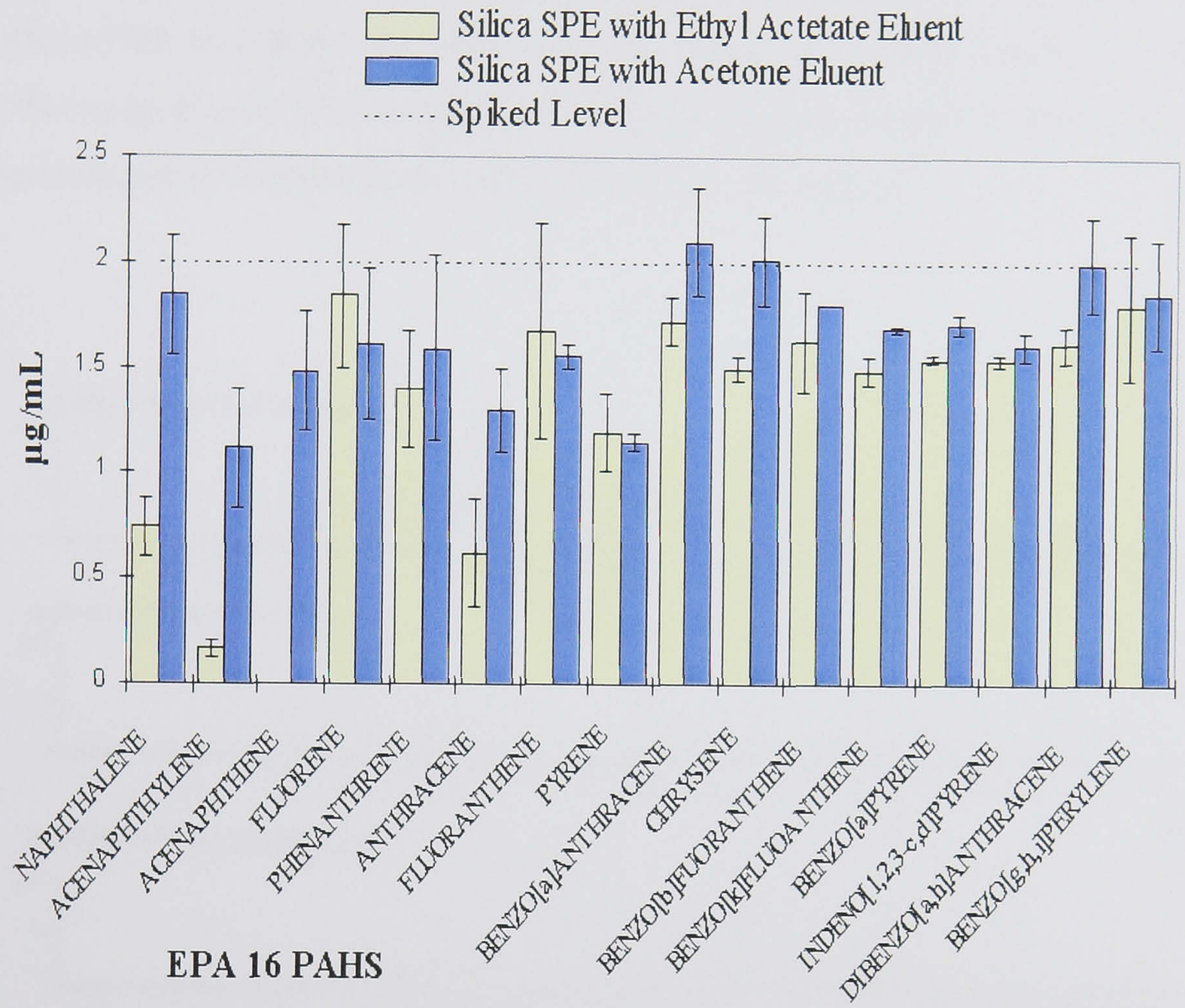


FIGURE 4.2. Spiked white oil (2 µg mL^{-1}) elution efficiency of acetone compared to ethyl acetate with silica solid phase extraction. SIC quantification of the EPA 16 priority PAHs was performed using Turbomass software.

4.2.2.2 Silica Sorbent Activation

The first approach examined was the optimisation of the SiO_2 SPE procedure, as SiO_2 is widely referred to in the literature as the most suitable sorbent for PAHs in oil (Thurman and Mills, 1998). Activating the SiO_2 by removing the water content improves PAH adsorption. However, a balance is required between adsorption and ease of elution. SiO_2 was dried overnight in a drying cabinet at 50°C to activate the

silica and improve the binding of polar substances. Filtration tubes with frits in the bottom were filled with silica (Section 2.2.4.3) and after conditioning with 8 mL cyclohexane, 50 μL of Nytro-10GBN was added. A 6 mL volume of cyclohexane was washed through until the fluorescence band was displaced to approximately one quarter of the way down the tube. A 6 mL volume of acetone was then used to elute the fluorescent band. The cyclohexane wash and acetone eluent fraction were collected separately and concentrated to 1 mL prior to GC-MS analysis.

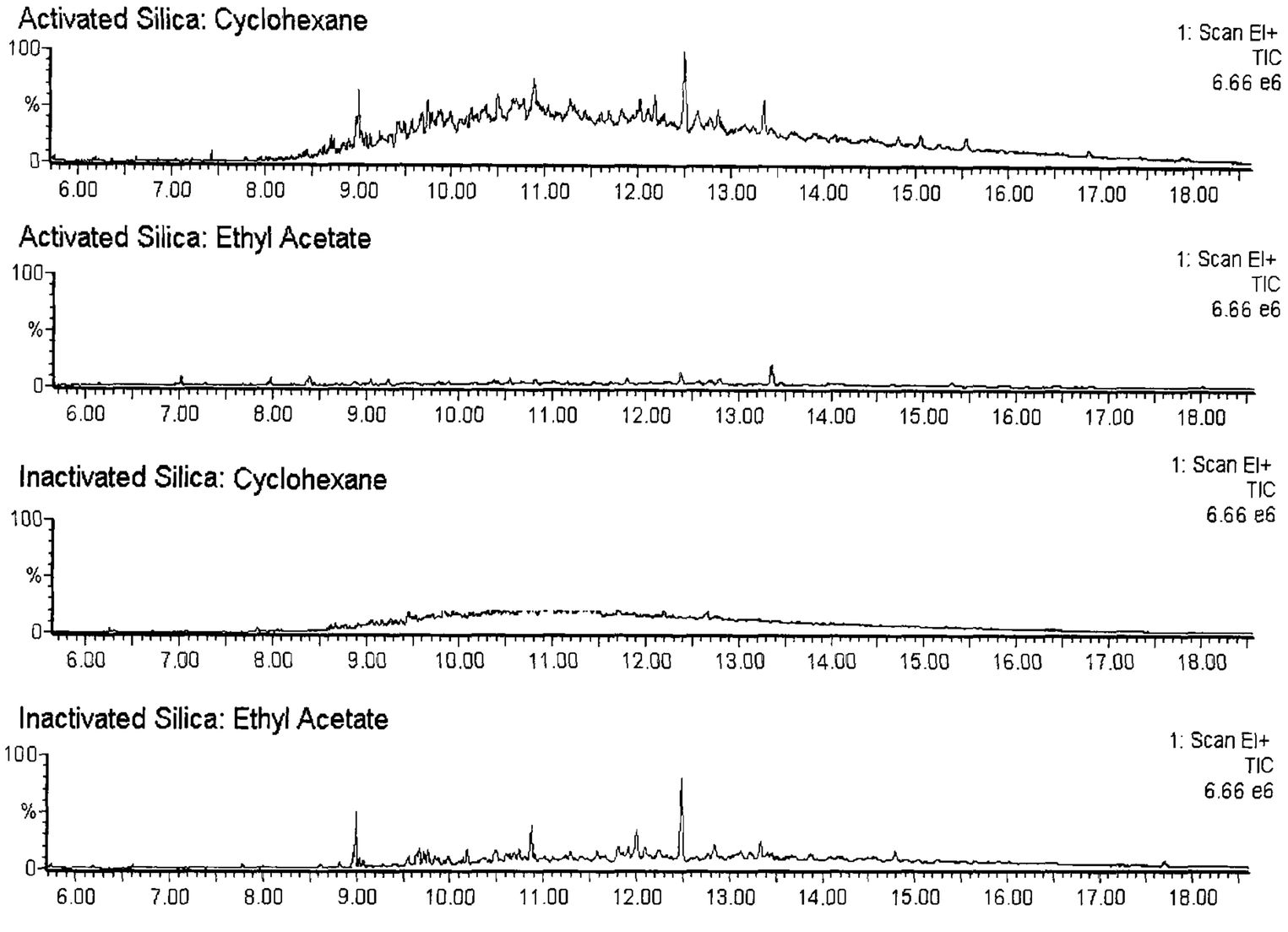


FIGURE 4.3. TIC comparison of the cyclohexane washes and ethyl acetate eluent of Nytro-10GBN from columns using activated and inactivated silica. Although no PAHs were identified from this TIC, it suggested that activated silica led to ineffective PAH elution due to the few peaks. This was confirmed with SIC.

Figure 4.3 shows the activated silica was better at removing unwanted components in the cyclohexane wash but PAH recovery was poor, presumably due to strong PAH adsorption to the sorbent. The cyclohexane wash with inactivated silica was not as

successful at removing unwanted components. However, the inactivated silica produced a TIC with better defined peaks, although specific PAHs could not be identified without selective ion monitoring (data not shown). The silica was therefore used without drying, under ambient laboratory conditions.

4.2.2.3 Variation on Column Dimension

An investigation was performed to determine the effect of column dimensions on extraction efficiency (Section 1.5.3.2). Washing required optimisation to purify oil extracts and hence produce accurate identification and quantification of PAHs. Figure 4.4 shows the results of an experiment performed with unspiked Nytro-10GBN on SiO_2 columns of two different lengths and diameters. The first was a 10 mm x 460 mm glass column. The column was filled with 2.5 g silica and washed with 5 mL cyclohexane. This extraction was repeated in the same column with 5 g sorbent and 10 mL cyclohexane. The remainder of the extraction process was performed as described in Section 2.2.4.1. The column dimension used in this experiment are summarised in Table 4.2.

The second column had twice the diameter of the first (20 mm x 200 mm). Once again 2.5 g silica was added, but due to its larger diameter, the column length was shorter than the previous column. This column was washed with 5 mL cyclohexane. The second column was then repeated with 5 g of silica and washed with 10 mL cyclohexane.

The second column (20 mm x 200 mm) cleaned the oil effectively, but the fluorescent band reached the end of the column with a lower volume of cyclohexane than in the 10 mm x 460 mm column so could not be cleaned with more than 5 mL of cyclohexane. The more sorbent that was used, the greater the volume of cyclohexane that could be added. Doubling the mass of sorbent therefore improved the sample clean up in both columns. However, the 10 mm x 460 mm glass column was more effective that the 20 mm x 200 mm column, when both contained the same amount of sorbent. This is best

illustrated in Figure 4.4 when the 20 mm x 200 mm with 2.5 g of sorbent (A) is compared with the equivalent 10 mm x 460 mm column (B). It was concluded that simply increasing the amount of sorbent was an improvement but maximising the sorbent bed length (and plate number), by reducing the column diameter, further improved cleaning. Having a small diameter to length ratio therefore allows optimisation of the washing step without losing large quantities of PAHs in the process and may improve PAH identification by removing interference. This correlates with plate theory that states that increased plate number improves efficiency (Thurman and Mills, 2000).

TABLE 4.2. Column dimensions investigated to determine the impact on extraction efficiency. Columns refers to chromatograms in Figure 4.4.

Column	*Length of Column (mm)*	*Diameter of Column (mm)*	*Sorbent Mass (g)*	*Volume of Cyclohexane Wash (mL)*
A	200	20	2.5	5
B	460	10	2.5	5
C	200	20	5	10
D	460	10	5	10

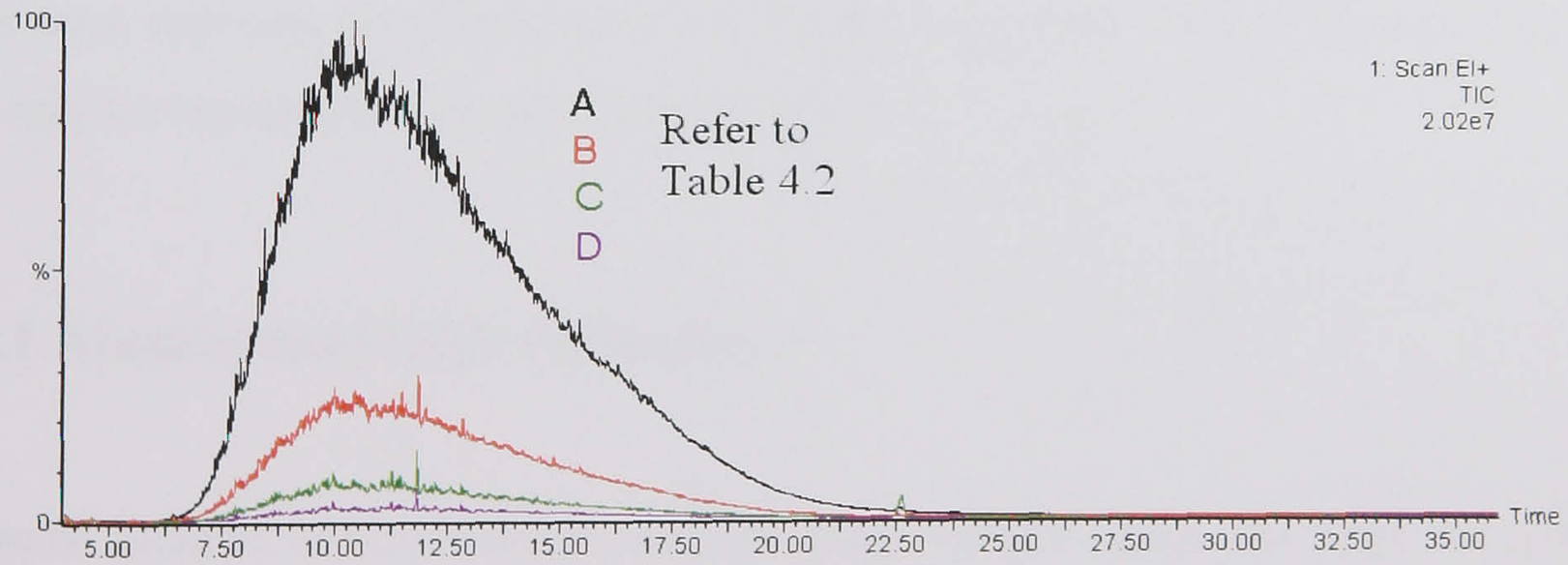


FIGURE 4.4. TIC of silica 10 mm x 460 mm column compared to a 20 mm x 200 mm column with Nytro-10GBN.

4.2.2.4 Flow Rate

As flow rate can significantly alter extraction efficiency (Section 1.5.3.2) the optimum flow rate of washing and elution solvents was investigated. The 10 mm x 460 mm column with 15 g of SiO_2 sorbent was washed with cyclohexane and eluted with acetone as in Section 2.2.4.1 at various flow rates. An appropriate flow rate for the wash solvent was established by determining (with an UV lamp) a flow that reduced fluorescent band broadening on the column. A washing flow rate of 2-3 mL min^{-1} was evaluated as a suitable compromise between band broadening and speed of extraction. The elution flow rate was established in the same way as the wash flow rate. It was observed that ~10% more solvent was required to elute the fluorescent band at a flow rate of 1 mL min^{-1} compared to 5 mL min^{-1}. This was possibly a consequence of reduced pressure through the column. Elution flow rates of 4-5 mL min-1 was therefore chosen to optimise the speed of extraction and reduce eluent volume.

4.3 ALTERNATIVE SORBENTS

Various sorbents were investigated to determine if they provided an alternative to SiO_2, or could be used with SiO_2 with further development. The sorbents were chosen because of their common use in the literature (Al, cyanopropyl, C18) or because they have been specially developed for PAH extraction in water or soil (Isolute PAH HC) and may be manipulated for use in an oil matrix.

4.3.1 Aluminium Oxide (Alumina)

Alumina (Al_2O_3) is an alternative normal phase sorbent commonly used for aromatic extraction from oils. However Figure 4.5 shows that Al_2O_3 has similar extraction efficiency to SiO_2 for the EPA 16 priority PAHs.

SiO_2 was more efficient at extracting the carcinogenic PAHs, and had better repeatability for fluorene, pyrene and benzo[g,h,i]perylene, so was the preferred sorbent. The improved repeatability of the SiO_2 method may be explained by Figure 4.6 which shows that silica's TIC chromatogram was cleaner and had better defined peaks than that of Al_2O_3.

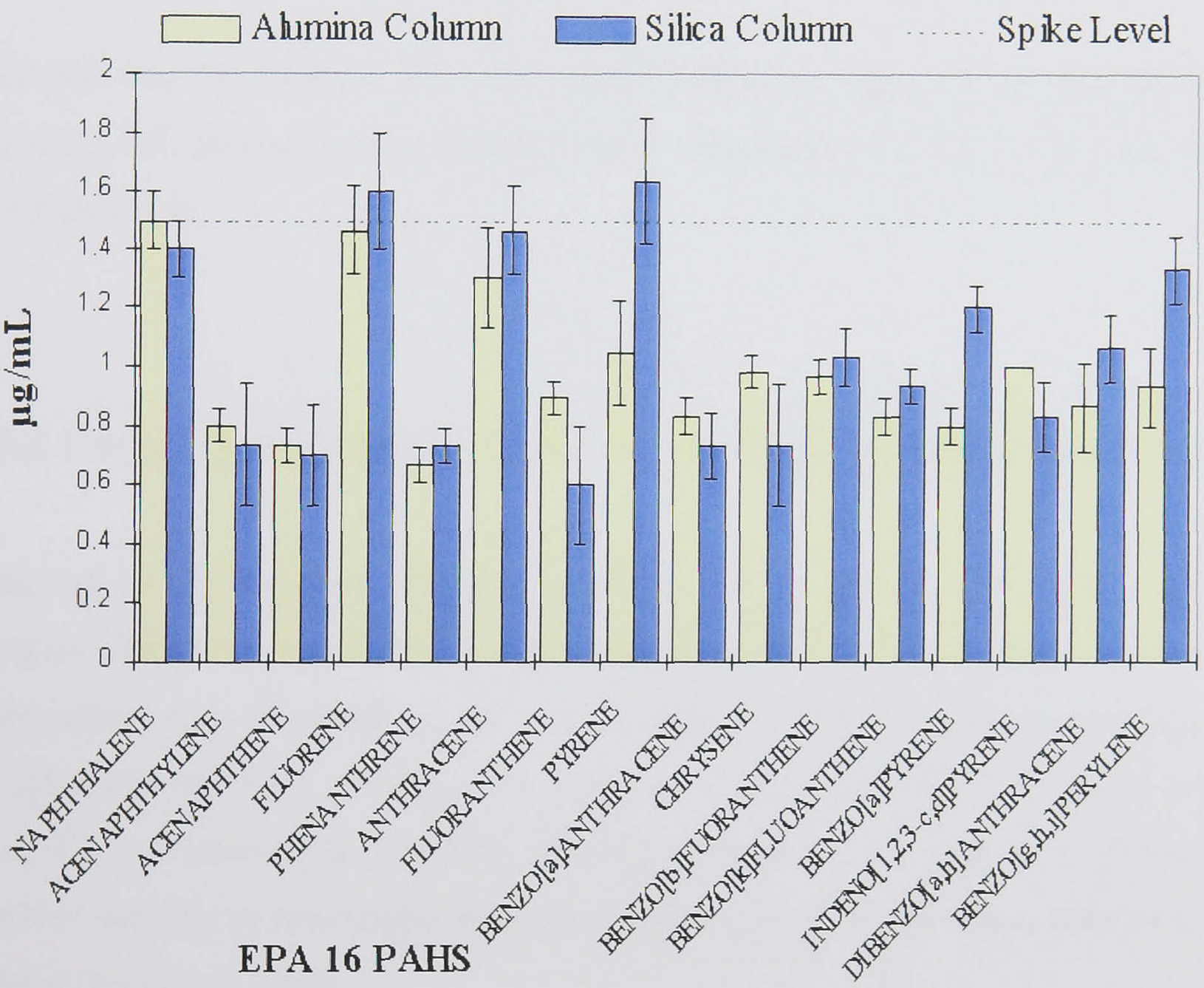


FIGURE 4.5. Extraction efficiency of spiked white oil (1.5 µg mL^{-1}) through a solid phase extraction with silica or alumina as the sorbent. SIC quantification of the EPA 16 priority PAHs was performed using Turbomass software.

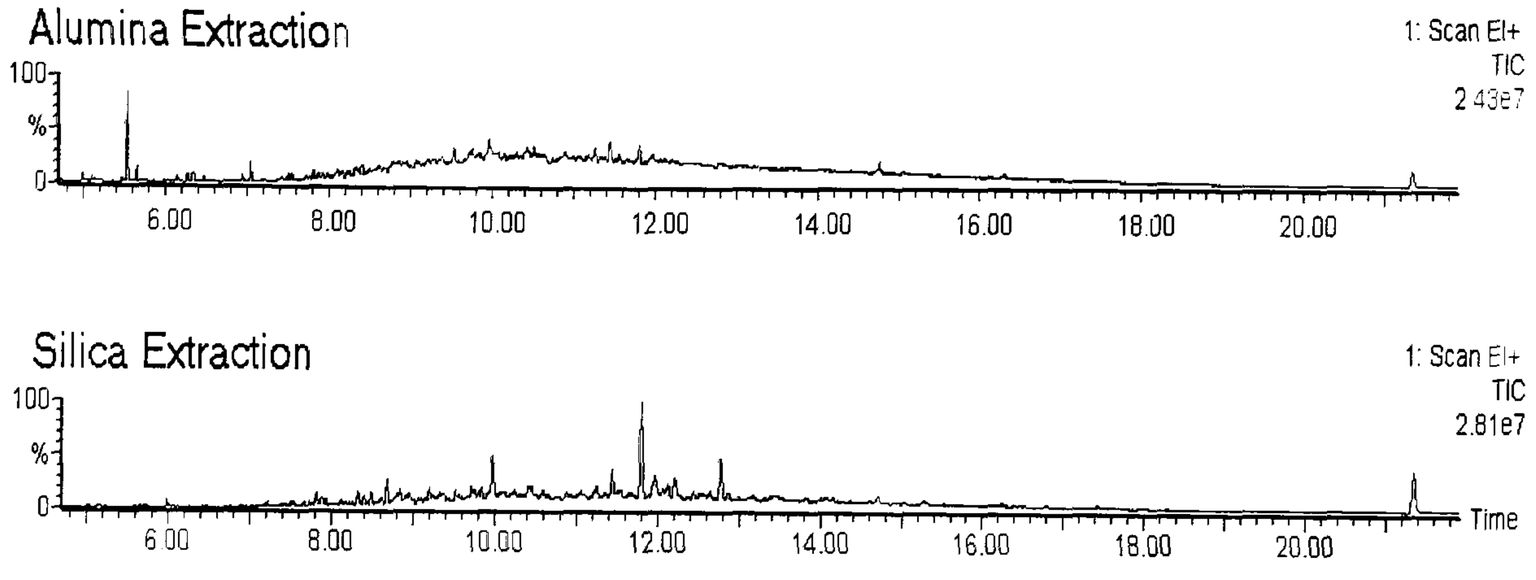


FIGURE 4.6. TIC spectra of Nytro-10GBN extracted with Alumina and Silica SPE. The silica TIC produced more defined peaks, suggesting that it may the better sorbent for oil clean-up.

4.3.2 Use of Cyanopropyl Sorbent as an Extra Sample Clean-Up Step

Referring to the literature, cyanopropyl was used pre-silica for the clean up of oil matrices (Thurman and Mills, 1998). Small-scale extraction columns were used as cyanopropyl was available as 900 mg cartridges. The cyanopropyl cartridge was placed above the SiO_2 column. The small scale SiO_2 columns were made in-house using 1.2 g of sorbent in a smaller diameter column 10 mm x 30 mm (greater plate number) than the commercially available columns (20 mm x 60 mm) containing 1 g of sorbent (Section 2.2.4.3).

After pre-conditioning both sorbents with 10 mL cyclohexane, 100 µL of spiked Nytro-10GBN was added to the top of the cyanopropyl cartridge. The sorbents were washed with 10 mL cyclohexane added to the cyanopropyl cartridge so that the fluorescent band entered the SiO_2 column and was washed through until it was approximately three quarters of the way down the SiO_2 column. The cyanopropyl cartridge was then removed and the fluorescent band was eluted with 6 mL acetone.

A SiO_2 column was also run without cyanopropyl, for a direct comparison. The oil was added directly to the SiO_2 column, and only 5 mL cyclohexane was required to wash the fluorescence down to the base of the column.

There was an improvement in extraction performance with the addition of the cyanopropyl step observed as more distinct peaks on the TIC (Figure 4.7). However, these peaks were not identified as PAHs from the MS spectrum and a substantial amount of interfering background material remained.

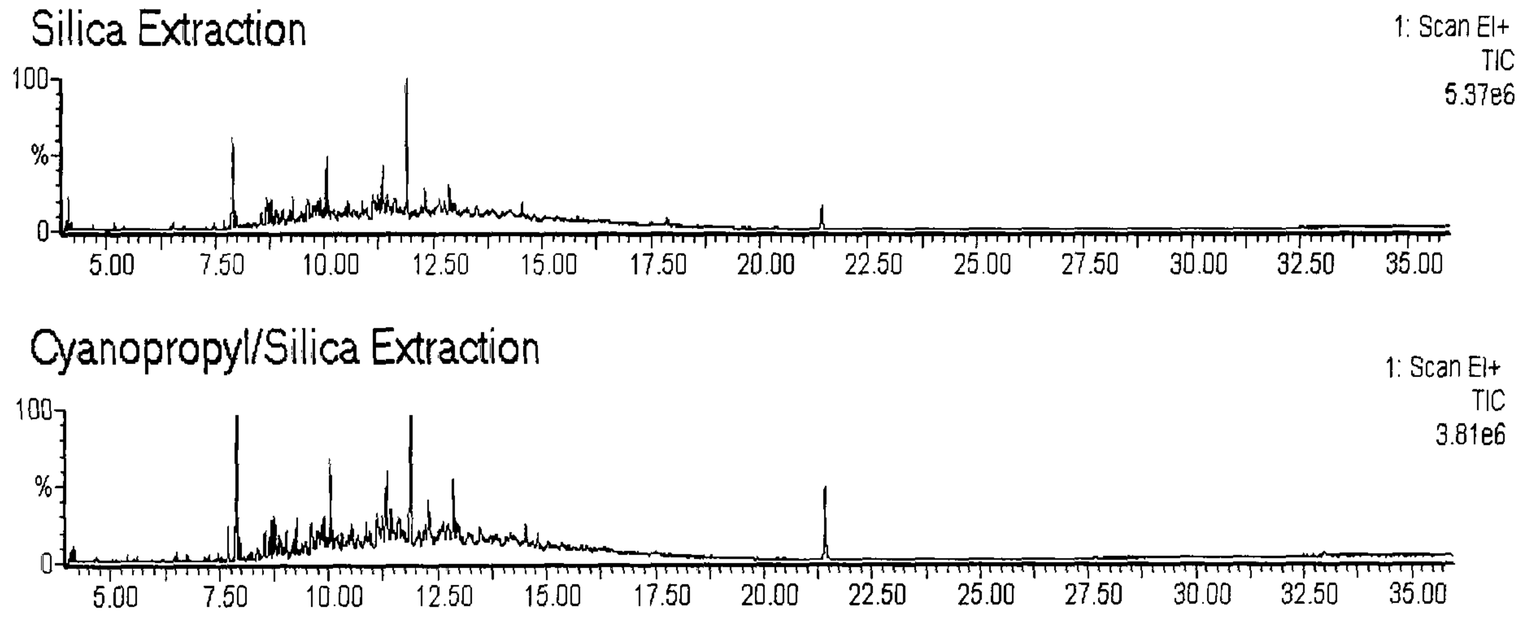


FIGURE 4.7. TIC of cyanopropyl/silica extraction compared to a silica extraction performed at the same time with Nytro-10GBN. The TIC shows vary little variation when a cyanopropyl sorbent is used pre-silica suggesting that there is no benefit in the extra step.

Figure 4.8 shows the extraction efficiency of the SIC cyanpropyl/SiO_2 method. It was clear that the addition of the cyanopropyl column did not dramatically improve PAH extraction. No significant differences were observed in extraction efficacy between the SiO_2 method and the cyanopropyl- SiO_2 method, therefore this approach was not examined further.

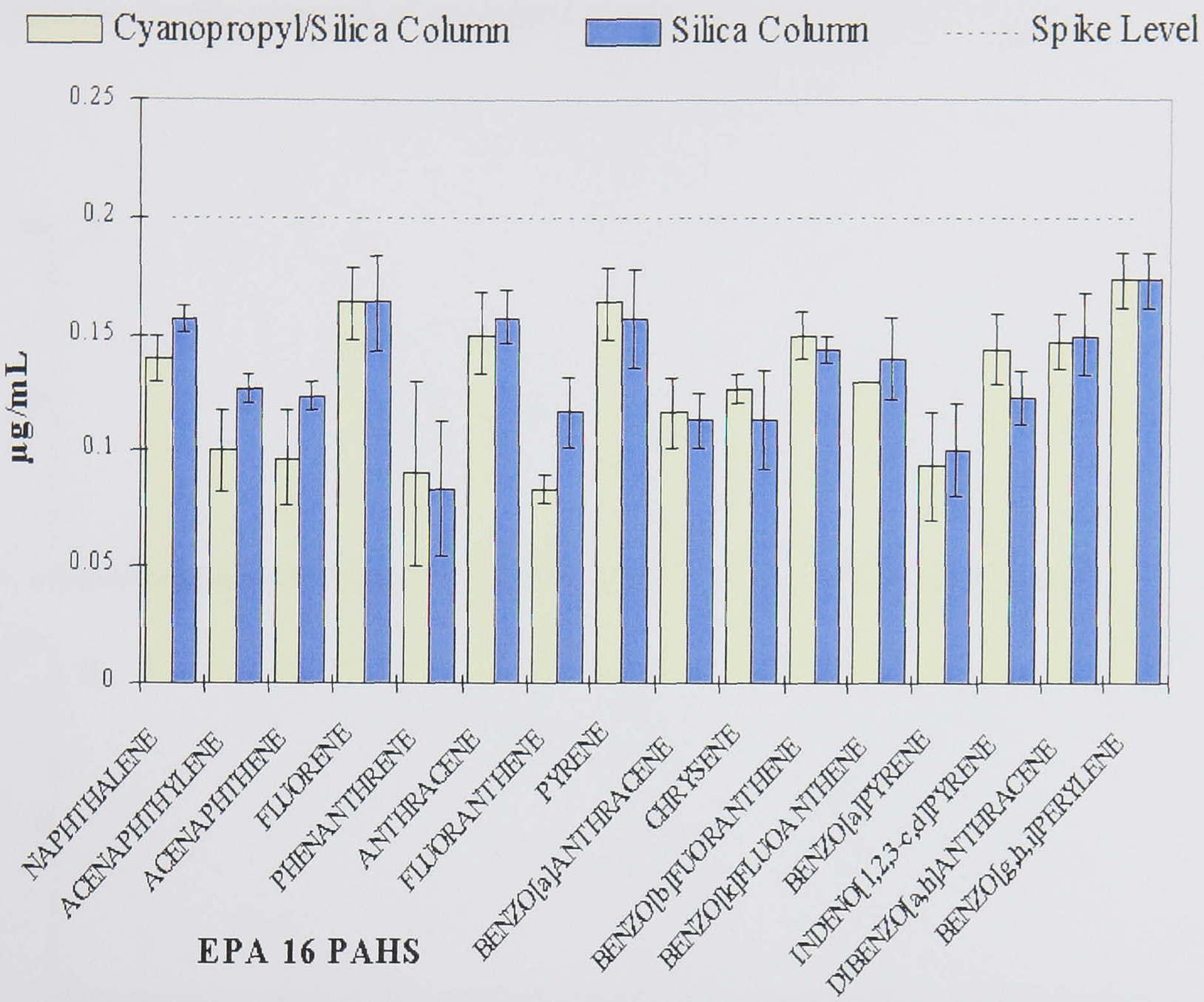


FIGURE 4.8. Extraction efficiency of spiked N10GBN oil (2 µg mL^{-1}) through a cyanopropyl/silica column compared to a silica column. SIC quantification of the EPA 16 priority PAHs was performed using Turbomass software.

4.3.3 Isolute PAH HC Column

A commercially available SPE cartridge, Isolute PAH HC was investigated for oil PAH extraction efficiency. The column was developed for extracting PAHs from water and soil. Jones Chromatography would not reveal the sorbents content of the column, but it was recommended for use with samples in organic solvents, so was compatible with oils. The extraction was performed with Nytro-10GBN, once with hexane as in the protocol, and once with cyclohexane, the less hazardous solvent preferred by the National Grid Company Plc. The protocol supplied with the column was used and is

outlined in Section 2.2.7.1 The TIC of each was obtained to determine the efficacy of the column for the removal of unwanted components from the oil (Figure 4.9).

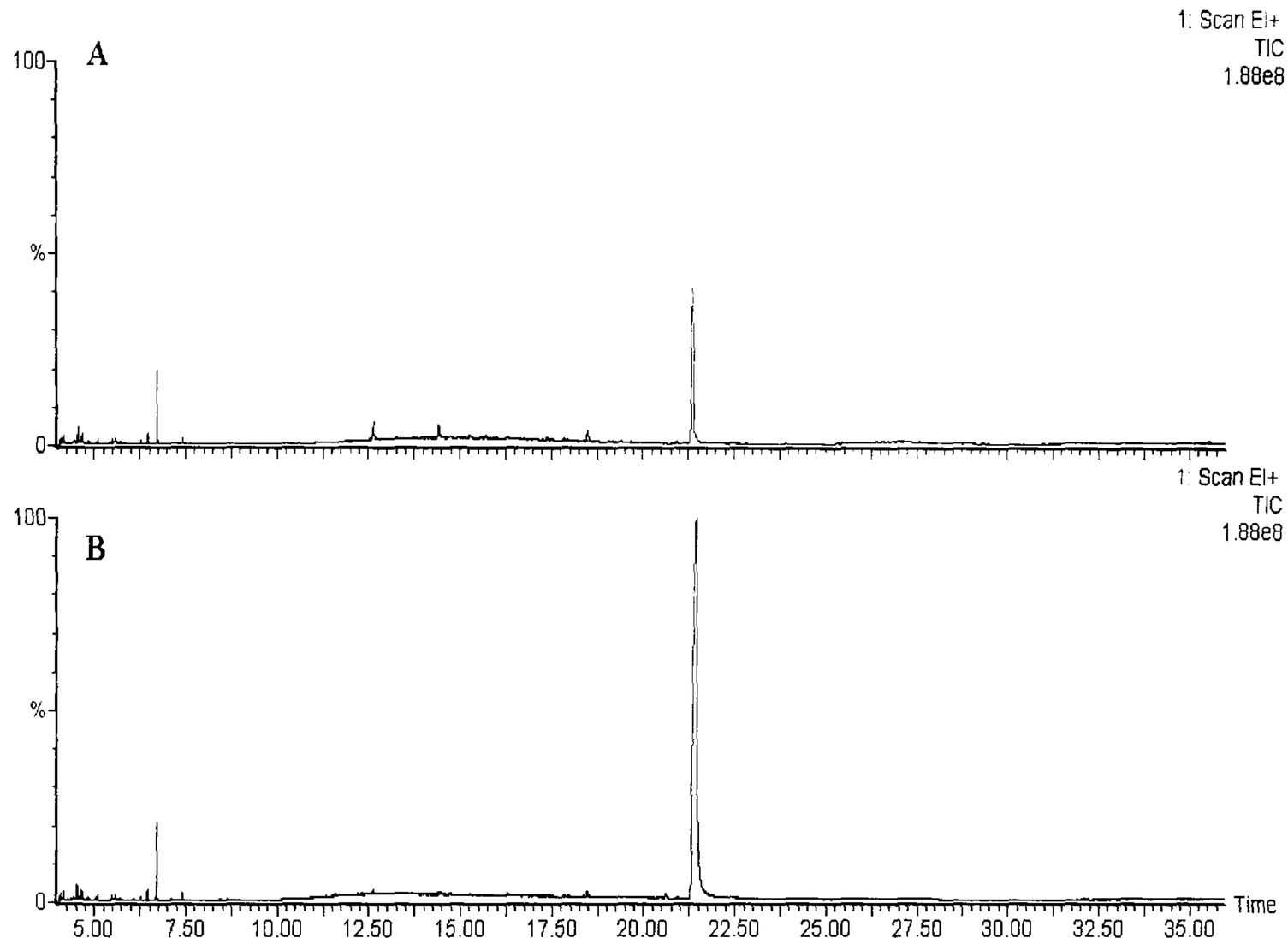


FIGURE 4.9. The TIC of oil Nytro-10GBN extracted with an Isolute PAH HC column using hexane (A) and cyclohexane (B). Both solvents produced a bare chromatogram, suggesting that PAHs were not extracted with this sorbent.

The chromatogram produced seemed bare, as if all interference had been removed, but no PAHs could be detected. Neither solvent proved more efficient at extracting. Therefore the extraction was repeated with white oil spiked with EPA 16 priority PAHs (2 $\mu g\ mL^{-1}$ each PAH) using cyclohexane and the extraction efficiency was determined from a SIC (Figure 4.10).

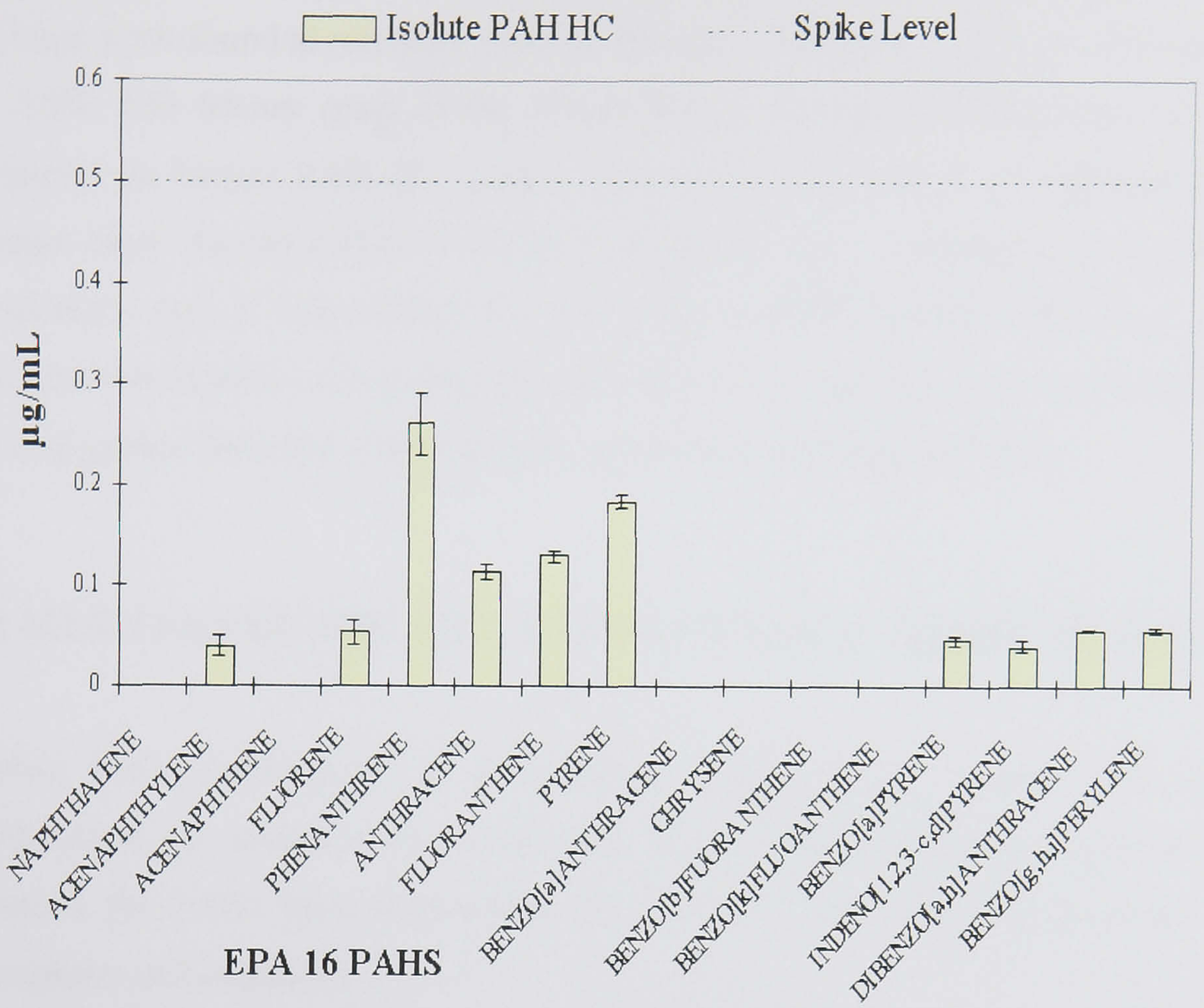


FIGURE 4.10. Extraction efficiency of EPA 16 priority PAHs spiked into white oil (2 µg mL^{-1}) using an Isolute PAH HC column. SIC quantification of the EPA 16 priority PAHs was performed using Turbomass software.

Extraction was very poor for all PAHs. However, the column is specifically manufactured for PAH extraction in soil and water so it was possible that the oil matrix was too complex for the column and an initial clean-up step would improve the efficiency of the column. The waste oil eluted from the column, although too complex to analyse by GC-MS, exhibited fluorescence suggesting that the oil was saturating the column therefore preventing full interaction between the sorbent and PAHs. This sorbent was therefore examined further as a final purification step following the development of a suitable preparative step (Section 4.5.1).

An important observation on the TIC of the Isolute PAH HC extract (Figure 4.9) was the large peak found at retention time 21 minutes. This peak could not be identified by the NIST MS library (part of the Perkin Elmer GC-MS software) but was present whenever an Isolute PAH HC column was used, so was likely to originate from the column itself. As the Isolute PAH HC column has been established as a useful final purification step, it was unlikely that this peak would be removed. However, although this peak would affect chromatogram scale due to its large size, it did not interfere with the data gained from the chromatogram so was not investigated further.

4.4 MULTI-STEP SPE FOR PAHS IDENTIFICATION IN OILS

Having been unsuccessful at extractions using a single sorbent, the use of a combination of sorbents was examined. Most clean-up protocols reported in the literature for PAHs from soils and water used only one sorbent for reasons of high throughput and simplicity.

Due the complex nature of the oil, it was unlikely that any one sorbent would remove sufficient interferents. The classic extraction method used in the literature involved a solvent clean-up followed by silica SPE (Wang, 2000). A similar method had already been performed (Section 3.7) and considering the large amount of solvent and time involved, as well as the inconclusive results gained with the Ames test, it was not considered an ideal method and would require considerable method development.

The fingerprinting technique developed at the National Grid Company Plc was an extension of the LLE/SPE method, which used a C18 column as an additional cleaning step (Wilson and Pahlavanpour, 2000). This method was useful as it provided a characteristic GC chromatogram that could be used to classify oil according to their aromatic content, and PAHs have been identified with this method by the National Grid Company Plc for Nytro-10GBN (Pahlavanpour and Wilson, 1999).

However this method was qualitative and not quantitative and this extract still contained many components other than PAHs. More importantly to this work, the

chromatogram exhibited numerous interferent peaks at later retention times, the region in which the carcinogenic PAHs would be eluted. As the work here was concerned with the measurement of PAH mutagenicity, it required a method that would minimise the presence of unwanted components at this later time in the chromatogram. This method was still useful however, as it provided important information on sorbent selection and experimental protocol.

4.4.1 Oil Fingerprinting

In the fingerprinting technique (Wilson and Pahlavanpour, 2000) three sequential extraction methods were used, to provide a cleaner PAH preparation (Section 2.2.8). There were however, disadvantages to the fingerprinting technique. These included the requirement for a large amount of oil (5 g) and as there were three separate extraction methods, losses from transfer and evaporation of solvent could occur. The method was also time consuming; the LLE alone took 2 hours to perform, particularly when the oils were aged, to ensure proper separation.

Figure 4.11 shows the fingerprinting technique TIC of oil 8, Nytro-10GBN and oil 4. The TIC for each oil showed a similar trend to IP 346, with the oil 8 extract having a greater mass of components when compared to Nytro-10GBN and in turn oil 4. However, the TIC clearly shows that these extracts were still too complex, and would not be suitable for Ames testing. However, the use of a reverse phase C18 extraction (as opposed to normal phase) introduced an alternative approach to extracting oils. It was postulated that this sorbent could prove useful in conjunction with SiO_2, LLE, or an Isolute PAH HC columns. The use of cyclohexane with the C18 sorbent in the fingerprinting technique created a non-polar environment in both the solid and liquid phases, rather than the polar and non-polar environment present in the silica/cyclohexane step and could offer improvements in sample clean-up.

FIGURE 4.11. TIC of oil 8, Nytro-10GBN and oil 4 using the mineral insulating oil fingerprinting technique. The initial large peaks (RT 5-7 minutes) are due to column bleed. The emergence of individual peaks is clearest for Nytro-10GBN but none of these peaks were conclusively identified as PAHs due to interference from other components in the oil.

The C18 sorbent was tested with whole oil using cyclohexane as the eluting solvent (as in the fingerprinting technique). Cyclohexane is a relatively non-polar solvent, but was suitable for PAHs elution as indicated by the progress of the fluorescent band. The resulting extract was still complex (Figure 4.12) but as the aromatic fraction was not retained on the column, the C18 could be useful as an initial crude extraction step and an alternative to the preliminary liquid-liquid extraction step.

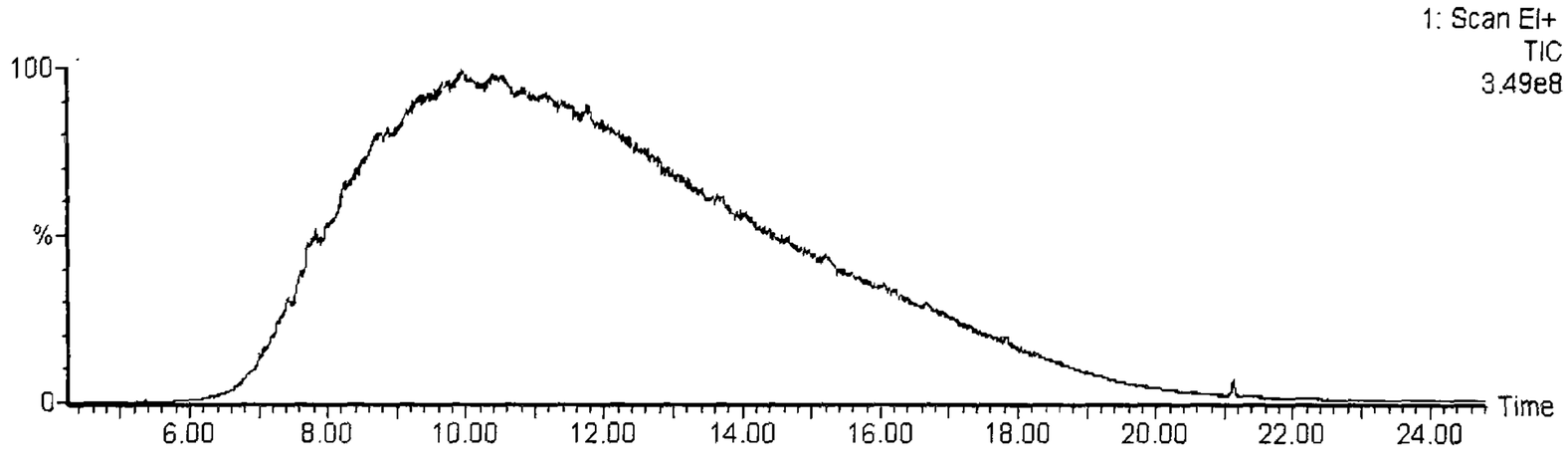


FIGURE 4.12. TIC of Oil Nytro-10GBN removed from a C18 column with cyclohexane. No defined peaks are detectable due to many interferent components still present, suggesting that C18 sorbent extraction alone is not effective at PAH extraction.

4.5 DEVELOPMENT OF THE SPE C18/SILICA/ISOLUTE PAH HC METHOD FOR PAHS

The fingerprinting technique, whilst having important diagnostic benefits was not suitable as a quantitative PAH analysis method. In addition, SPE methods were preferable to LLE, as they used less solvent and by their nature are less hazardous to health. They also allowed fractionation of a sample, which may be useful for separating oil components that have very similar bulk properties. SPE can also be automated. An additional benefit of using SPE over LLE is that PAHs fluoresce and therefore can be followed through SPE using an UV lamp. This prevented column overload and allowed simple optimisation of washing volume, to avoid analyte loss. For this reason, the main emphasis on method development was in the area of SPE. However, LLE were not ruled out at this stage as it may serve a valuable role in combination with other methods, as found in the fingerprinting protocol.

As a number of different combinations of sorbent were used, the TIC proved particularly useful as a rapid indicator of interferent removal and PAH identification. If an extraction proved promising with TIC the extraction efficiency was determined using SIC.

Although primary development involved a trial and error approach to combining sorbents, the work to date gave clues to the best extraction combinations. The main requirement of the extraction was to recover PAHs with the least amount of interferent components. However, other considerations were considered important. The method should preferably minimise solvent volume and use less hazardous solvents. Ideally, it should be more rapid than existing methods, and be simple to perform. These extra concerns were addressed once the main requirement had been fulfilled. As with most procedures, some compromises had to be made in order to meet as many of the criteria as possible.

The work to date indicated two promising sorbents. The first was the Isolute PAH HC column, said to be excellent for isolating PAHs by the manufacturer in less complex matrices (soil and water). This column was therefore used as a final purification step after sufficient quantities of the unwanted components were removed by an appropriate clean-up step. C18 sorbent was another possibility, but as a facilitator for sample clean-up.

4.5.1 Liquid-Liquid Extraction Followed by Isolute PAH HC Column

The first method performed was based on the LLE/SPE combination reported so frequently in the literature (Grimmer *et al.*, 1981; Wang, 2000). The Isolute PAH HC column was used after LLE to determine if a reduction in matrix complexity improved the Isolute PAH HC purification.

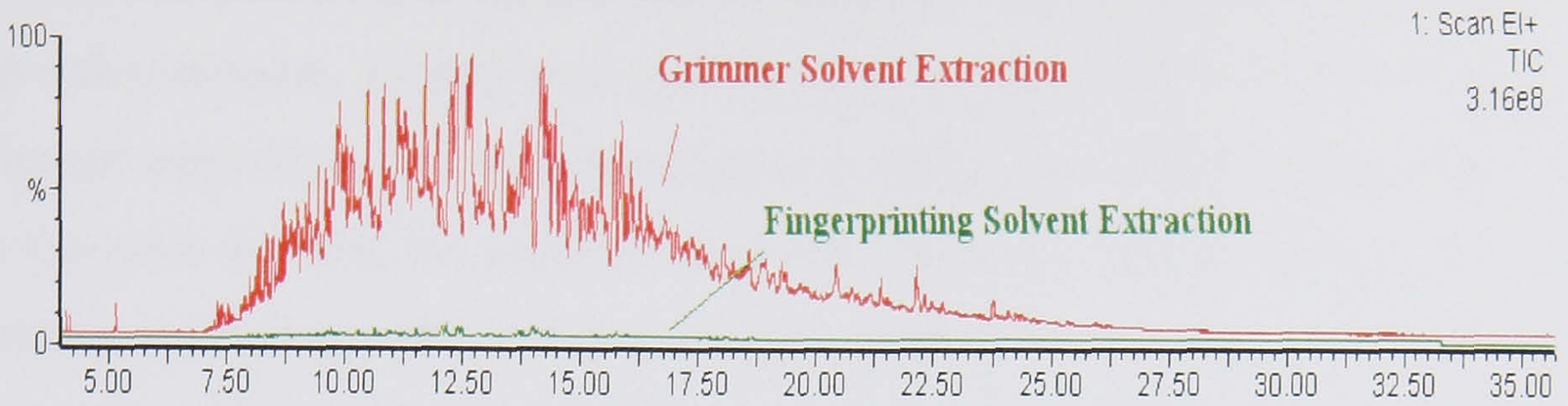


FIGURE 4.13. The TIC of oil 8 with Grimmer Liquid-liquid extraction compared to the liquid-liquid extraction in Wilson and Pahlavanpour (2000) prior to passage through an Isolute PAH HC column.

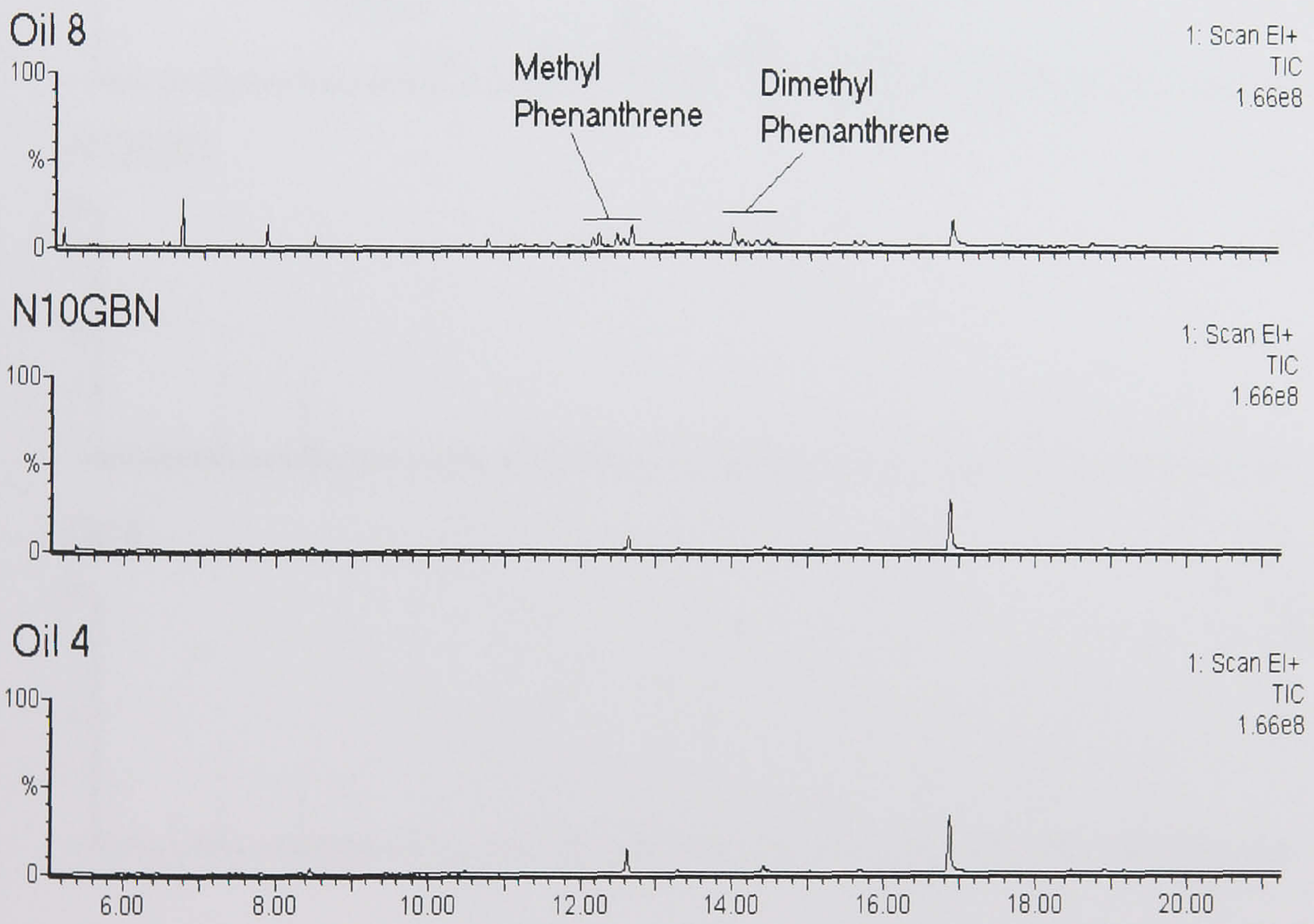


FIGURE 4.14. The TIC of the liquid-liquid extraction in Wilson and Pahlavanpour (2000) followed by Isolute PAH HC. Oil 8 native PAHs are labelled.

The LLE method used in the mineral oil fingerprinting technique developed by Wilson and Pahlavanpour, (2000) was chosen as an initial clean up step along with the Grimmer extraction. The fingerprinting LLE method worked on the same principles as the Grimmer method, but used five times the volume of solvent in each step for further cleaning, producing a less complex extract, as illustrated in Figure 4.13. The modified protocol used for the Isolute PAH HC extraction is described in Section 2.2.7.2.

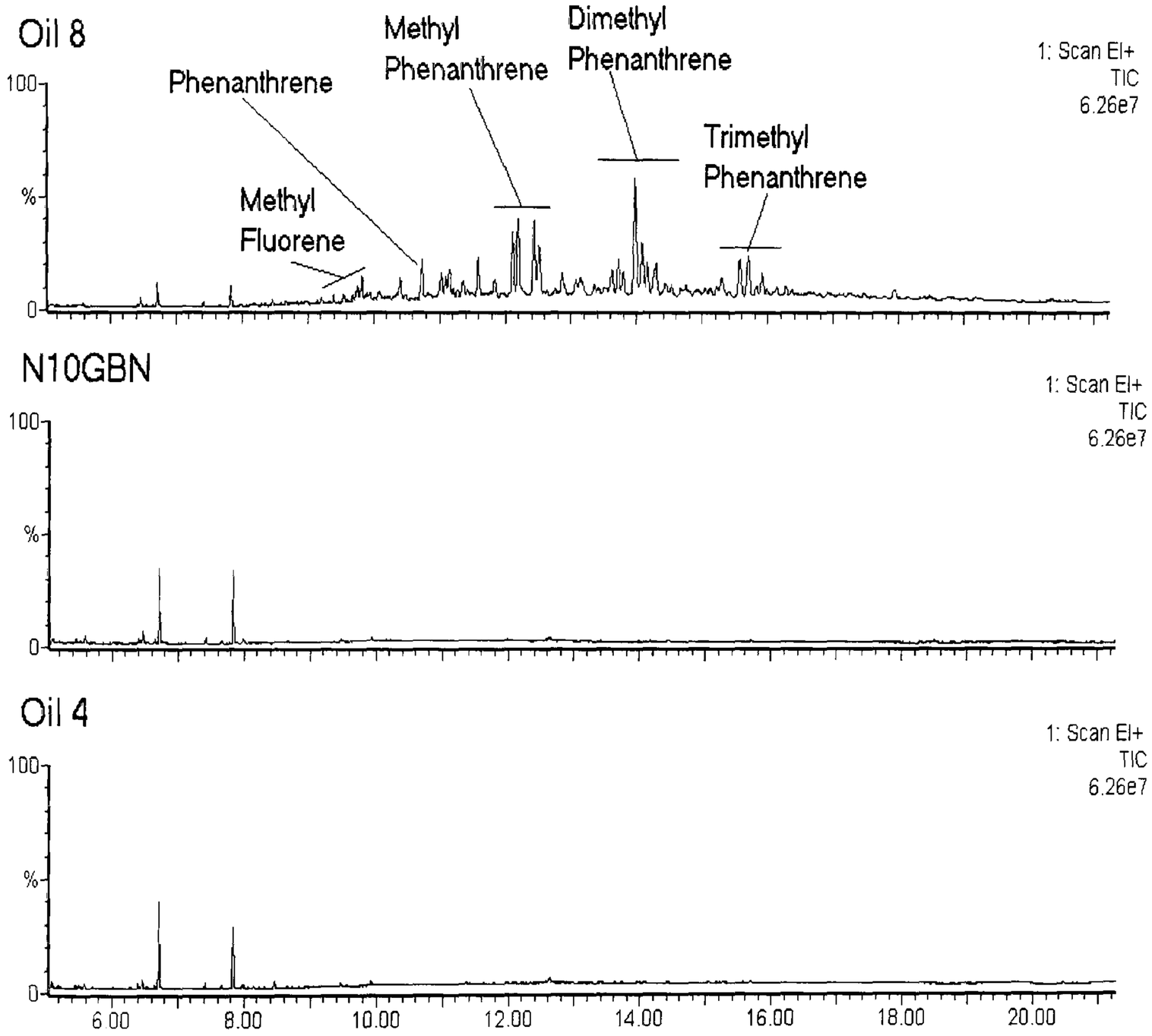


FIGURE 4.15. The TIC of Grimmer liquid-liquid extraction followed by Isolute PAH HC of oil 8, Nytro-10GBN and oil 4. Oil 8 native PAHs are labelled.

The fingerprinting LLE with Isolute PAH HC purification indicated a low level of PAHs in oil 8 only (Figure 4.14). Improved extraction was noted for the combined Grimmer/Isolute PAH HC procedure (Figure 4.15) and showed a clean profile for oil 8 but with greater PAH recoveries. However, like the fingerprinting LLE, oil 4 and Nytro-10GBN gave featureless chromatograms.

As oil 8 contained a greater aromatic content than the other two oils, the results suggested that larger quantities of oil 4 and Nytro-10GBN were required for GC-MS analysis. However, as 5 g of oil was already being used in the LLE, the use of more oil became impractical. For this reason, this extraction method was not developed further. Ideally, a method that does not involve LLE would be preferable, as it would not only use less oil and solvent, it would also be simpler to perform and more repeatable. Nevertheless, the Isolute PAH HC column may offer benefits as a final purification step, and with a better clean-up method may produce a clean extract ideal for PAH identification and Ames testing.

4.5.2 C18 with Isolute PAH HC

The C18 column showed promise as an additional method for oil extract clean-up and so was performed in combination with the Isolute PAH HC column to determine if the C18 sorbent could replace the LLE step. A loading of 100 μL of oil was used on a 900 mg C18 cartridge. Conditioning with 10 mL cyclohexane occurred prior to Nytro-10GBN oil loading and 3 mL cyclohexane was added, displacing the fluorescence onto the Isolute PAH HC column. The Isolute PAH HC column was then treated as in the LLE/Isolute PAH HC purification method (2.2.7.2).

Figure 4.16 shows the TIC of Nytro-10GBN extracted by the C18/Isolute PAH HC method. The chromatogram was very similar to that of the single stage Isolute PAH HC extraction. During the procedure, the column appeared overloaded with extract. The fluorescence was spread throughout the column, rather than in a band as with the LLE/Isolute PAH HC method. However, reducing the oil load did not improve the

TIC. It was possible therefore, that the extract required further clean-up before it was added to the Isolute PAH HC column.

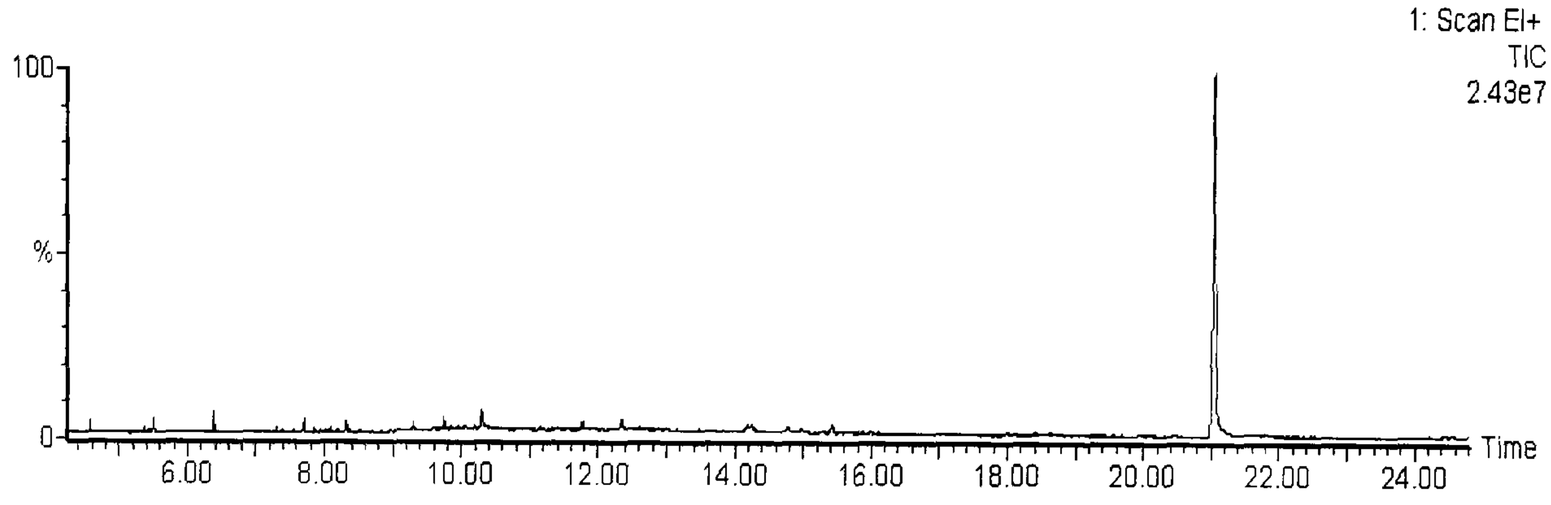


FIGURE 4.16. TIC of Nytro-10GBN from C18 clean-up followed by Isolute PAH HC purification. No peaks were identified as PAHs.

4.5.3 Combined C18-Silica SPE for Oil Clean-Up

As the C18 sorbent appeared ineffective in cleaning the oil sample prior to loading onto the Isolute PAH HC, a SiO_2 column was used for further clean-up. The SiO_2 and C18 sorbents were used in combination to determine which order offered the best clean-up method.

The silica column was used prior to C18 by extracting a 100 µL sample of oil as in Section 2.2.4.3 and adding the final extract (after acetone evaporation) to a conditioned 900 mg C18 cartridge in 1 mL cyclohexane. The extract was eluted from the C18 sorbent in 3 mL cyclohexane and evaporated to 1 mL for GC-MS analysis.

The addition of a silica clean-up step (Figure 4.17) improved the extraction procedure compared to the TIC of C18 only (Figure 4.12). However, using C18 before SiO_2 (as described in Section 2.2.9) had a further cleaning effect as shown in Figure 4.18. Peaks were more visible with the C18/SiO_2 extract, although individual PAHs could not be identified. The C18/ SiO_2 clean-up was therefore used before the Isolute PAH HC column, to ascertain if this combination produced a cleaner extract.

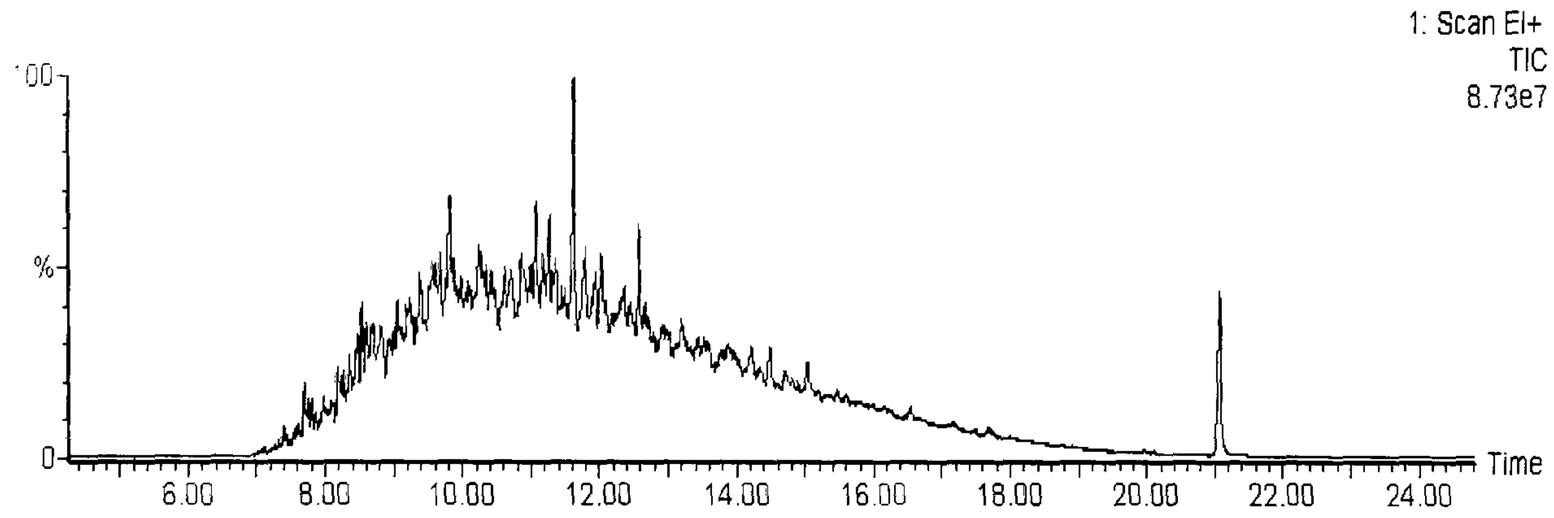


FIGURE 4.17. TIC of Nytro-10GBN clean-up with silica followed by C18 sorbent.

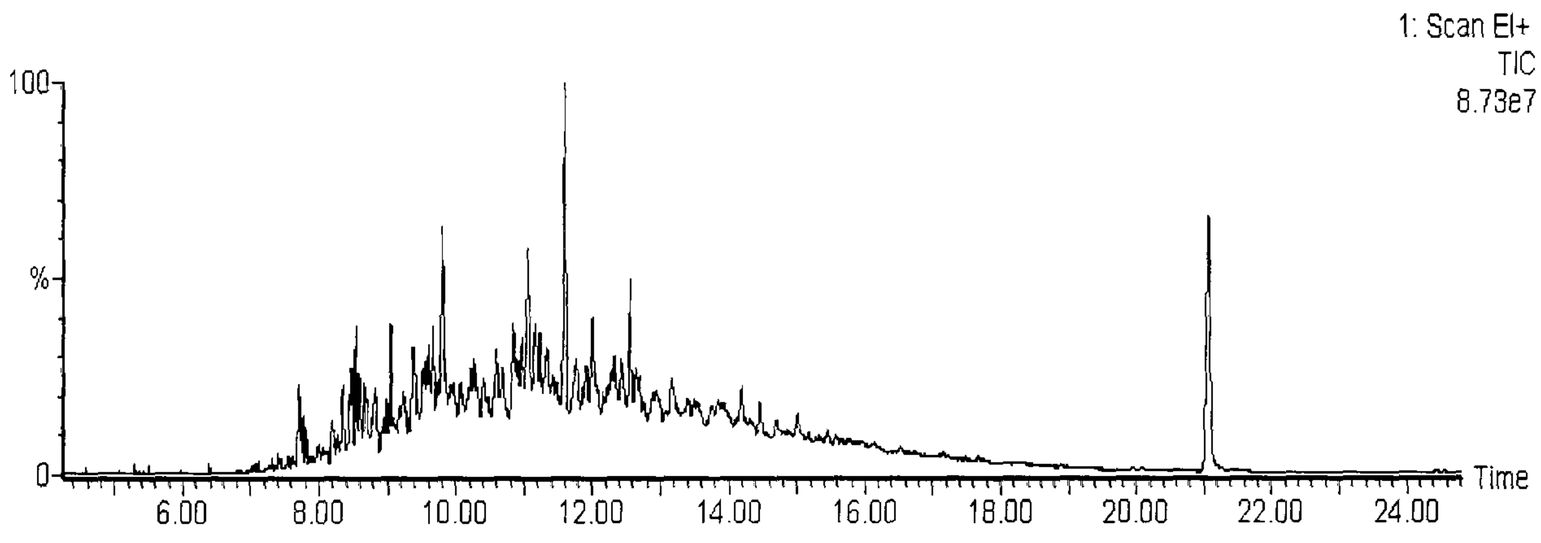


FIGURE 4.18. TIC of Nytro-10GBN clean-up with silica followed by C18 sorbent.

4.5.4 C18/Silica/Isolute PAH HC

The C18/SiO_2 method was combined with the Isolute PAH HC method and is detailed in Section 2.2.9. The oil used for extraction was Nytro-10GBN spiked with 2 μgmL^{-1} of each EPA 16 priority PAH. The spike allowed the extraction to be evaluated in regard to identifying PAHs from the TIC, as it would indicate if these PAHs were visible without resorting to SIC. Figure 4.19 shows the TIC of spiked Nytro-10GBN and shows both PAHs spiked into the oil and native species. It was possible that the presence of so many native PAHs acted to obscure the spiked species at retention times 10-16 minutes. However, a number of higher molecular weight PAHs spiked into the

oil were visible due to minimal interferences at later retention times. This method seems to be more specific to PAHs than the previous methods performed.

4.6 TESTING THE C18/SILICA/ISOLUTE PAH HC EXTRACTION FOR EFFICIENCY AND REPEATABILITY

The C18/Silica/Isolute PAH HC method was effective at removing unwanted components from the oil extracts. However, the repeatability of this method had to be tested, along with extraction efficiency.

The extraction efficiency was measured with white oil and Nytro-GBN by quantifying the recoveries of PAHs from spiked and unspiked oil samples. Quantification was achieved by SIC detection, followed by manual integration, and comparison with a calibration curve over 6 concentrations at a range of 0.05-2 $\mu g\ mL^{-1}$ (each repeated in duplicate). Each extraction was performed 6 times per oil for both the spiked and unspiked samples. Table 4.3 contains the calibration data. The %CV of these plots, shows that there was between 4.1-11.8% deviation in the calibration curve slope. No imprecision was recorded for the intercept as the trend line was forced through zero (Miller and Miller, 2000).

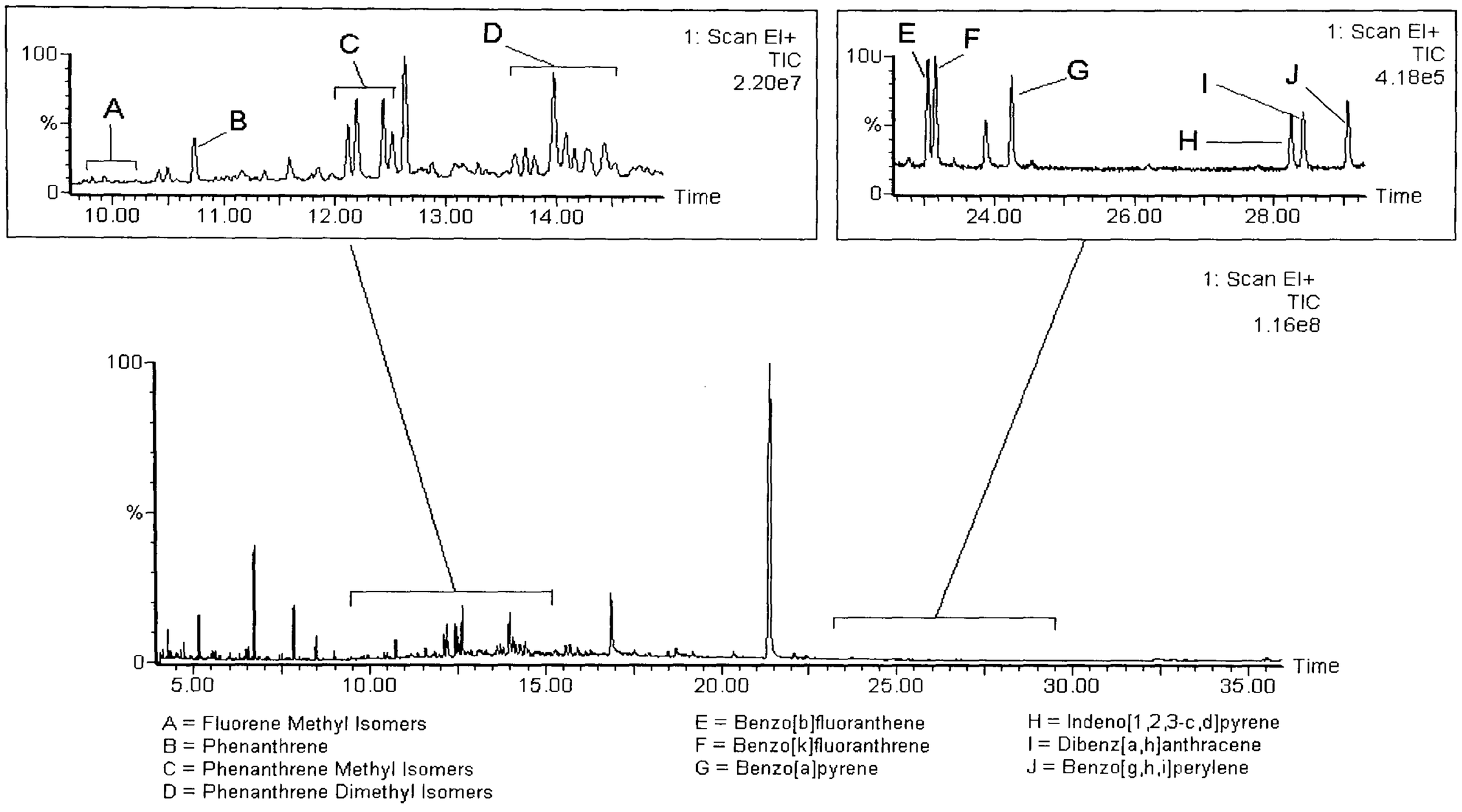


FIGURE 4.19. TIC Scan for N10GBN Transformer Oil spiked with 2 μgmL^{-1} extracted by C18 Silica PAH HC Isolute method showing close up of PAHs identified in oil. A-D: naturally occurring PAHs, E-J: EPA PAHs spiked into oil.

TABLE 4.3. Calibration data including slope deviation and limit of detection for quantification of the EPA 16 priority PAHs spiked into oil.

PAH Spiked into Oil	*Retention Time*	R^2	*Graph Equation*	*Error for Slope (%)*	*Calculated Limit of Detection (μgmL^{-1})*
Naphthalene	5.962	0.9946	y = 399414x	7	0.049
Acenaphthylene	7.469	0.9959	y = 450051x	9	0.020
Acenaphthene	7.682	0.9944	y = 275767x	8	0.19
Fluorene	8.411	0.9922	y = 274641x	10	0.067
Phenanthrene	10.311	0.9964	y = 417115x	7	0.093
Anthracene	10.431	0.9715	y = 325691x	11	0.29
Fluoranthene	13.851	0.9983	y = 416997x	4	0.13
Pyrene	14.628	0.9928	y = 402271x	9	0.0093
Benzo(a)anthracene	19.508	0.9881	y = 183947x	10	0.10
Chrysene	19.663	0.9888	y = 189378x	11	0.021
Benzo(b)fluoranthene	23.98	0.9920	y = 137052x	6	0.11
Benzo(k)fluoranthene	24.085	0.9850	y = 119160x	12	0.13
Benzo(a)pyrene	25.207	0.9947	y = 110893x	8	0.14
Indeno(1,2,3-c,d)pyrene	29.246	0.9852	y = 93853x	8	0.21
Dibenz(a,h)anthracene	29.408	0.9878	y = 79794x	12	0.017
Benzo(g,h,i)perylene	30.072	0.9878	y = 111758x	9	0.0057

Tables 4.4 and 4.5 show the concentrations of both native EPA 16 priority PAHs and those spiked into white oil and Nytro10GBN. By subtracting the concentration values of the naturally occurring PAHs from the equivalent spiked PAHs, the extraction efficiency of the method for the spiked PAHs was determined. The repeatability of the extraction method is shown as %CV values.

As previously measured by SiO_2 and Al SPE, unspiked white oil contained no detectable PAHs as the values in Table 4.4 were below the limits of detection calculated in Table 4.3. The most common EPA 16 priority PAHs in the Nytro-10GBN oil were fluorene, phenanthrene, anthracene and pyrene.

Tables 4.4 and 4.5 also shows that the extraction efficiencies for those EPA 16 priority PAHs identified as probable human carcinogens by the IARC (benzo[a]anthracene to dibenzo[a,h]anthracene) were 69.3-89.8% for the two types of oil tested. Of the remaining PAHs, the extraction efficiency varied from 21.2-71.4%, except for the most volatile PAHs (naphthalene, acenaphthylene and acenaphthene) where efficiency was ≤ 3.3%.

TABLE 4.4. Extraction efficiency of spiked white oil (2 μg mL^{-1}) and unspiked oil through a small scale C18/silica/Isolute PAH HC.

PAH	*White Oil Extract in Acetone (μgmL^{-1})*	*%CV White Oil*	*White Oil Spiked with 20 μg mL^{-1} EPA 16 Minus native PAHs (μgmL^{-1})*	*%CV of spiked White Oil*	*Percent Efficiency (%)*
Naphthalene	0	0	0.04	0	2
Acenaphthylene	0	0	0.06	0	3
Acenaphthene	0	0	0	0	0
Fluorene	0.05	0	0.4	15	21
Phenanthrene	0.06	0	1.1	5	58
Anthracene	0.06	3	0.8	18	43
Fluoranthene	0.05	0	1.3	11	67
Pyrene	0.06	21	1.2	6	60
Benzo(a)anthracene	0.04	0	1.6	8	84
Chrysene	0.05	22	1.5	6	78
Benzo(b)fluoranthene	0.04	0	1.5	10	79
Benzo(k)fluoranthene	0.04	0	1.6	12	81
Benzo(a)pyrene	0.04	0	1.6	4	83
Indeno(1,2,3-c,d)pyrene	0.03	0	1.3	10	69
Dibenz(a,h)anthracene	0.03	0	1.6	9	82
Benzo(g,h,i)perylene	0.03	6	1.3	9	65

TABLE 4.5. Extraction efficiency of spiked Nytro-10GBN oil (2 μg mL^{-1}) and unspiked oil through a small scale C18/silica/Isolute PAH HC.

PAH Spiked into Oil	*N10GBN Oil Extract in Acetone ($\mu g\ mL^{-1}$)*	*%CV of N10GBN*	*Nytro-10GBN Spiked with 20 $\mu g\ mL^{-1}$ EPA 16 Minus native PAHs (μgmL^{-1})*	*%CV of Spiked N10GBN*	*Percent Efficiency (%)*
Naphthalene	0	0	0	0	0
Acenaphthylene	0.06	0.7	0	36	0.04
Acenaphthene	0	0	0	0	0
Fluorene	0.10	14	0.4	28	23
Phenanthrene	0.12	10	1.1	8	59
Anthracene	0.09	17	0.9	20	49
Fluoranthene	0.07	0	1.4	15	71
Pyrene	0.37	41	1.0	12	50
Benzo(a)anthracene	0.04	0	1.5	19	78
Chrysene	0.07	0	1.5	8	78
Benzo(b)fluoranthene	0.04	0	1.6	13	81
Benzo(k)fluoranthene	0.04	0	1.7	10	86
Benzo(a)pyrene	0.04	0	1.7	13	90
Indeno(1,2,3-c,d)pyrene	0.03	0	1.4	6	75
Dibenz(a,h)anthracene	0.03	0	1.6	15	83
Benzo(g,h,i)perylene	0.03	0	1.3	11	68

The 2 oils give similar extraction efficiencies, which suggested that the greater complexity and higher aromatic content of the Nytro-10GBN transformer oil did not greatly affect the quantitative nature of the extraction method. However, overall repeatability was better for white oil, in which 11 of the 13 %CV values were lower than the corresponding Nytro-10GBN values. The C18/Silica/Isolute PAH HC extraction method is further discussed in Section 7.3.3.

4.6.1 Loss of PAHs from Evaporation

In order to determine the loss of PAHs during the evaporation steps, Nytro-10GBN prepared by the C18/Silica/Isolute PAH HC method was reconstituted in 9.9 mL cyclohexane and spiked with 0.1 mL EPA 16 priority PAHs in cyclohexane (1 $\mu g\ mL^{-1}$ per PAH), mixed and left to evaporate for 16 hours. The spiked extract was then reconstituted in 1 mL acetone and analysed by GC-MS. The experiment was performed at the same time as the previous extraction efficiency tests, to allow the calibration data in Table 4.3 to be used to quantify PAH losses during the evaporation step. Results are shown in Figure 4.20. The evaporation step was found to be the main cause of the low recoveries of the more volatile PAHs such as naphthalene (20%), acenaphthylene (11%), acenaphthene (0%), fluorene (27%), phenanthrene (61%) and anthracene (62%).

An extract of Nytro-10GBN was also spiked after extraction and subjected to rotary evaporation to see if this improved recovery of the more volatile PAHs (Figure 4.20). Naphthalene was not retained by rotary evaporation but recovery of acenaphthene, fluorene and phenanthrene improved by 10-20%, acenaphthylene by nearly 40% and almost complete anthracene recovery was achieved, but at a loss of assay precision. This is possibly due to the restricted amount of solvent (1-2 mL) used to recover the sample from the round bottom flask. Washing the flask with more solvent increased efficiency, but the extra solvent belies the purpose of the evaporation step. With such small quantities of sample, it was therefore concluded that evaporation at room temperature was more appropriate. Although greater losses of the volatile PAHs were incurred, it was more efficient at recovering the carcinogenic PAHs, as sample transfer was not necessary.

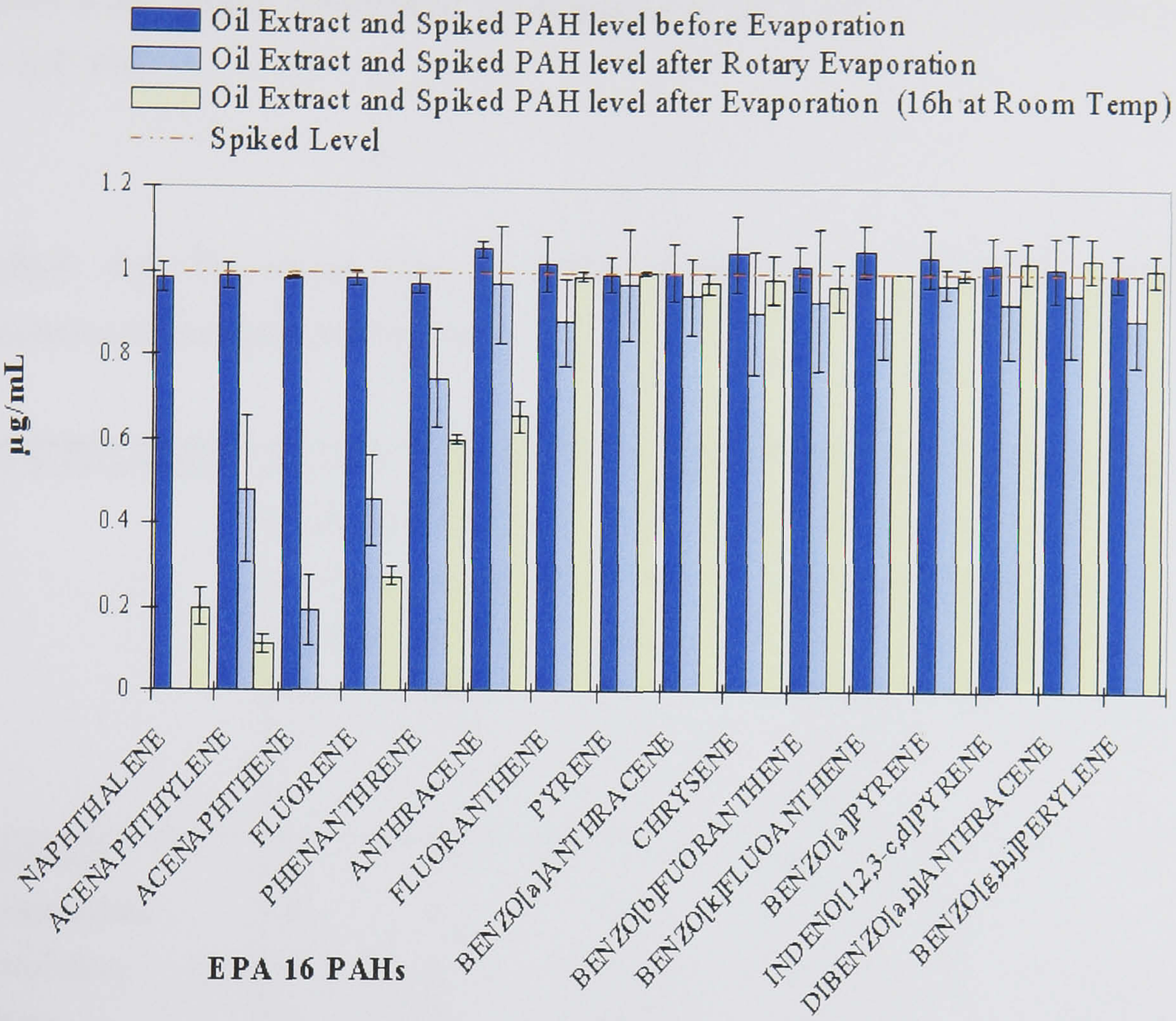


FIGURE 4.20. PAH losses during the C18/Silica/Isolute PAH HC extraction process. Shown are the results for Nytro-10GBN oil extract spiked with EPA 16 priority PAHs before evaporation and after both rotary evaporation and room temperature evaporation over 16 hours.

4.6.2 Total Loss of PAHs from the C18/Silica/Isolute PAH HC

Each stage of the extraction was analysed to determine exactly where losses of PAHs occurred. Results are shown in Table 4.6. Once again the quantification was performed at the same time as the calibration outlined in Table 4.3. Losses of PAHs during the C18 extraction were determined by passing 10 mL of acetone through the column to remove residual PAHs. Up to 18% losses of individual PAHs was found, the greatest loss being the larger, carcinogenic PAHs. PAH levels in the cyclohexane and pentane

washes were simply determined by evaporating the wash to 1 mL and analysing by GC-MC SIC.

TABLE 4.6. Percentage loss of PAHs throughout the extraction method and calculation of unaccounted species.

PAH Spiked into Oil	*Loss of PAHs from the C18 Column (%)*	*Loss of PAHs from Cyclohexane Wash (%)*	*Loss of PAHs from Pentane Wash (%)*	*Total PAHs Not Accounted for in Method for White Oil (%)*	*Total PAHs Not Accounted for in Method for N10GBN Oil (%)**
Naphthalene	0	15.1	0	3	5
Acenaphthylene	0	6.7	0	1	5
Acenaphthene	0	0	0	0	0
Fluorene	0	0	8.2	-2	-3
Phenanthrene	0	0	0	4	2
Anthracene	6.8	0	6.1	6	6
Fluoranthene	7.7	0	0	23	18
Pyrene	8.3	0	0	35	42
Benzo(a)anthracene	0	0	0	14	19
Chrysene	10.7	0	0	5	6
Benzo(b)fluoranthene	0	0	0	16	14
Benzo(k)fluoranthene	12.3	0	0	0	0
Benzo(a)pyrene	14.2	0	0	-1	0
Indeno(1,2,3-c,d)pyrene	0	0	0	31	26
Dibenz(a,h)anthracene	15.5	0	0	5	3
Benzo(g,h,i)perylene	17.2	0	0	18	15

**Total PAH not accounted for include losses from room temperature evaporation illustrated in Figure 4.22*

The final two columns of Table 4.6 reports the percentage of PAH not accounted for by these investigations. These losses were possibly due to PAHs being irreversibly sorbed to the column or due to experimental error. The amount of pyrene not accounted for (31-41%) was of particular concern, but as pyrene was not a carcinogenic PAH, no further attempts to improve recovery were made.

4.6.3 Extraction Efficiency over a Range of Spike Levels

Further determination of the quantitative nature of the method was accomplished by measuring PAH extraction efficiency across the EPA 16 priority PAH loading range of 0-4 $\mu g\ mL^{-1}$ (total PAH of up to 64 $\mu g\ mL^{-1}$) (Figure 4.21). This revealed the limits of the extraction process, indicating the point at which the extraction process becomes inefficient. Of the 16 EPA priority PAHs, 4 were measured for extraction efficiency; phenanthrene as a non-mutagenic PAH with intermediate extraction efficiency (58-60%), indeno[1,2,3-c,d]pyrene with a slightly better efficiency (70-75%) and benzo[b]fluoranthene, benzo[k]fluoranthene which had the best extraction efficiencies (79-86%) and represented the carcinogenic PAHs which were of greatest importance.

Figure 4.21 shows a linear relationship between extraction efficiency and analyte concentration across the range 0-4 $\mu g\ mL^{-1}$ per PAH and thus a promising quantitative method for carcinogenic PAHs. The result suggests that a single oil loading may be used (100 μL) during routine sample testing. However, confirmation of this supposition would be achieved by examining different oil loadings in cases where unrefined oil, aged oil or oil other than that used in transformers was under analysis.

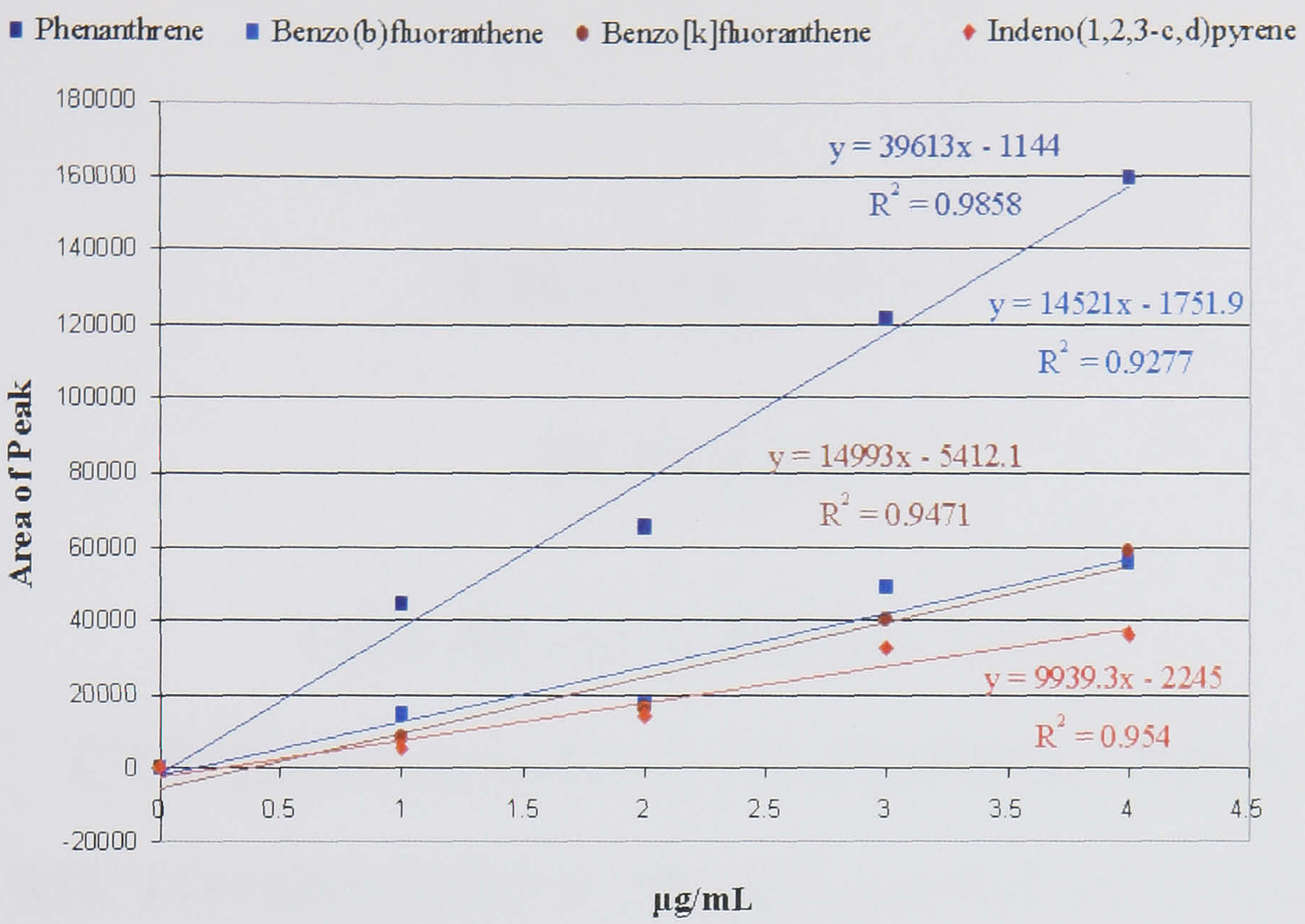


FIGURE 4.21. Extraction efficiency of phenanthrene, indeno[1,2,3-c,d]pyrene, benzo[b]fluoranthene and benzo[k]fluoranthene when added in a mixture with the EPA 16 priority PAHs at up to 4 µg mL^{-1} per PAH.

4.7 CONCLUSIONS

The efficiency and improved cleanness of the developed C18/Silica/Isolute PAH HC extraction method suggested that it would work better with the mutagenicity tests than previous methods and may overcome the problem of mixing the oil extract with the aqueous Ames test environment. However, this extraction method would require scaling up to produce enough extract to test at a variety of doses in the mutagenicity tests. This may prove to be difficult, for although the C18 and silica stages could be scaled up due to the commercial availability of both sorbents, the Isolute PAH HC column was only available in 1g cartridges. This could be overcome by running the C18/SiO_2 through several Isolute PAH HC columns, but would mean that the extraction became more time consuming. In addition, it was possible that by scaling up the procedure could become less efficient. It may therefore be of benefit to take a different approach to the problem and try to down scale the mutagenicity tests.

CHAPTER 5.0

RESULTS

THE BLACKBURN AND C18/SILICA/ISOLUTE EXTRACTION METHODS WITH MUTAGENICITY AND TOXICITY TESTS

5.1 INTRODUCTION

In chapter 4, an extraction method was developed that produced a significantly cleaner extract than previous extractions and proved efficient for oil loadings of up to 4 μg mL^{-1} of each EPA 16 priority PAH. As the Ames test required large amounts of extract to test mutagenicity at a range of doses, either the extraction method requires scale up, or the Ames test requires scale down. Both approaches offer benefits and drawbacks.

A larger scaled C18/silica/Isolute PAH HC extraction would not necessarily yield the same extraction efficiency, even if the volume of solvent, sorbent weight and column dimension remained proportionate. GC-MS analysis of the large scale extracts would require comparison to the small scale extracts and more optimisation was envisaged.

Scaling down of the Ames test would allow small scale extracts to be used with no more extraction development required. A small scale Ames test (Miniscreen) has been reported (Brooks, 1995), and thus was evaluate here with the oil extracts. This method still requires extensive preparation however and has a long incubation period. As the drawbacks of using the Miniscreen test were simpler to overcome than those associated with scaling up the extraction method, the Miniscreen test was investigated first (Section 5.3).

However, to validate the use of C18/Silica/Isolute PAH HC extracts with the Ames test, a further extraction method was simultaneously tested for mutagenicity. Previously, the Grimmer method had been performed using 80% v/v S-9, as described by Blackburn *et al.* (1984), a well accepted modification for testing oil mutagenicity in the literature (Brooks *et al*, 1995). The modified Ames test was now utilised in full, by combining the 80% v/v S-9 with the liquid-liquid extraction (LLE) that was later developed as part of the modified method (Blackburn *et al.,* 1986). This method will be referred to as the Blackburn extraction (Section 2.2.3.3). The Blackburn extraction therefore provided another extraction method for comparison with IP346, Grimmer and the C18/silica/Isolute PAH HC Ames test results.

5.2 AMES TESTING OF BLACKBURN EXTRACTS WITH 80% S-9

As the less time consuming Grimmer method proved to be better at indicating mutagenicity than the industry standard IP 346 method, a similar method, found to be successful with the Ames test was used. The method is a LLE method developed by Blackburn *et al.,* (1986) as part of the modified Ames test. Blackburn extracts of oils 8, 4, Nytro-10GBN and white oil were Ames tested at the same time as the C18/Silica/Isolute PAH HC purification to validate the new method. The revertant numbers found on individual Ames test plates are given in Appendix C. As described in Section 2.2.2, the dose of oil extract for Ames testing was given as milligrams of oil extracted (as opposed to milligrams of oil extract) per plate.

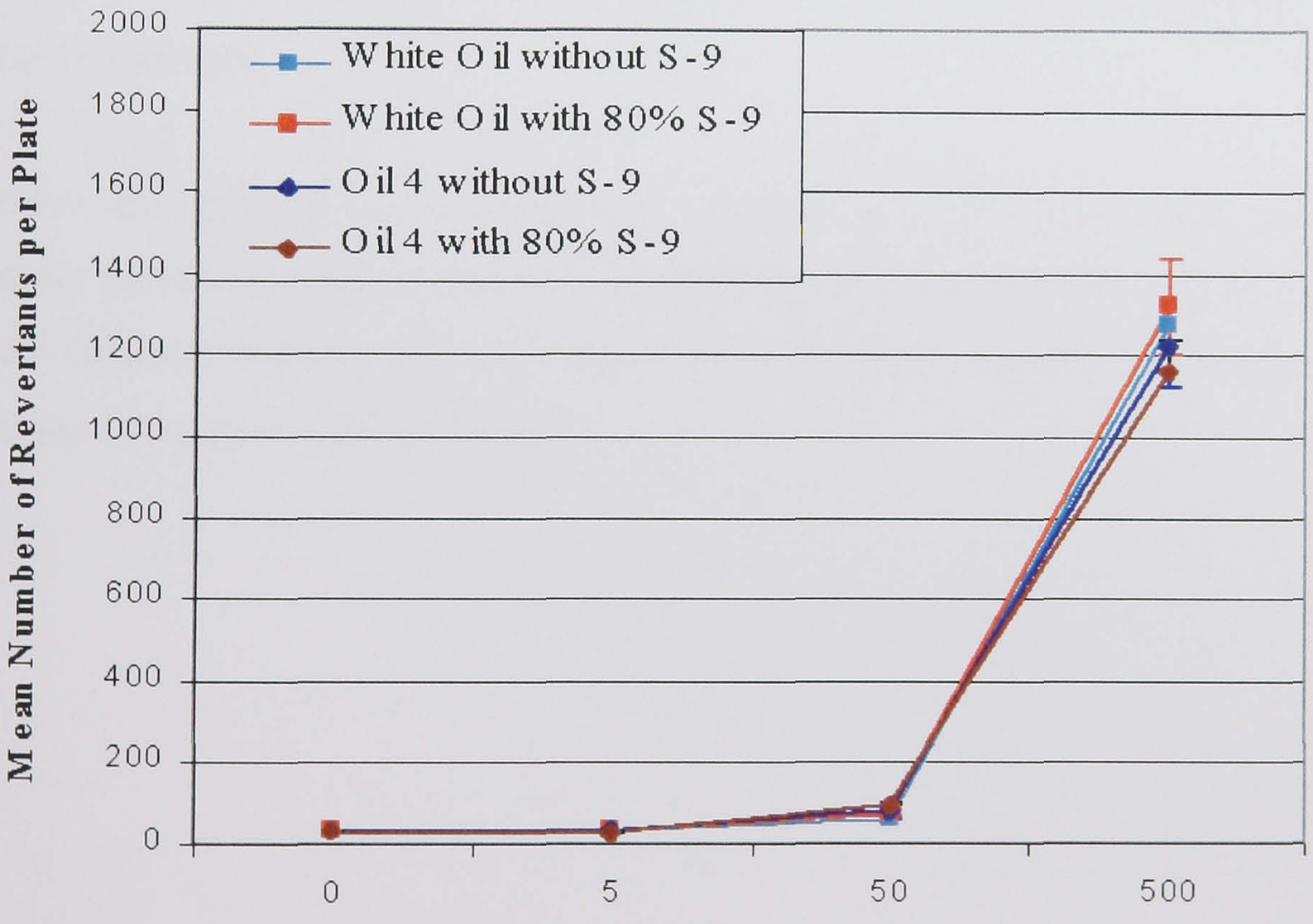


FIGURE 5.1. The Ames test revertants on the addition of Blackburn oil extracts of white oil and oil 4 over 4 doses in the absence and presence of S-9 (n = 3). %CV ranges from 3.6-24.7%.

Direct mutagenicity was observed for white oil, oil 4 (Figure 5.1), Nytro-10GBN and oil 8 (Figure 5.2) extracted by the Blackburn method at 500 mg plate^{-1}. Direct mutagenicity was believed to originate from simple aromatics extracted with the polyaromatics in liquid-liquid extraction (Section 1.1.3.1). Revertant numbers were extremely high as with the Grimmer extracts, as all oil extracts produced over 800 revertants with or without S-9. As with the Grimmer extracts, oil 4 and white oil indicated no indirect mutagenicity.

The Blackburn extract results correlated with the Grimmer results seen in Section 3.4.2 with a significant increase in mutagenicity observed on the addition of S-9 for both oil 8 and Nytro-10GBN. However, the Blackburn extracts for oil 8 and Nytro-10GBN registered a similar level of mutagenicity after activation (40%) whereas the Grimmer extracts had exhibited a 35% increase in revertants for Nytro-10GBN and 50% for oil 8. As IP 346 % w/w data had shown oil 8 to contain 3 times the aromatic content of Nytro-10GBN, the Grimmer extract mutagenicity showed a greater correlation with the industry standard.

Indirect mutagenicity (as with Grimmer extracts) was only observed at the highest dose for Blackburn extracts, but was validated by the presence of indirect mutagenicity in the Grimmer extracts and C18/Silica/Isolute PAH HC extracts. Results are further discussed in section 7.4.1 and 7.4.2.1.

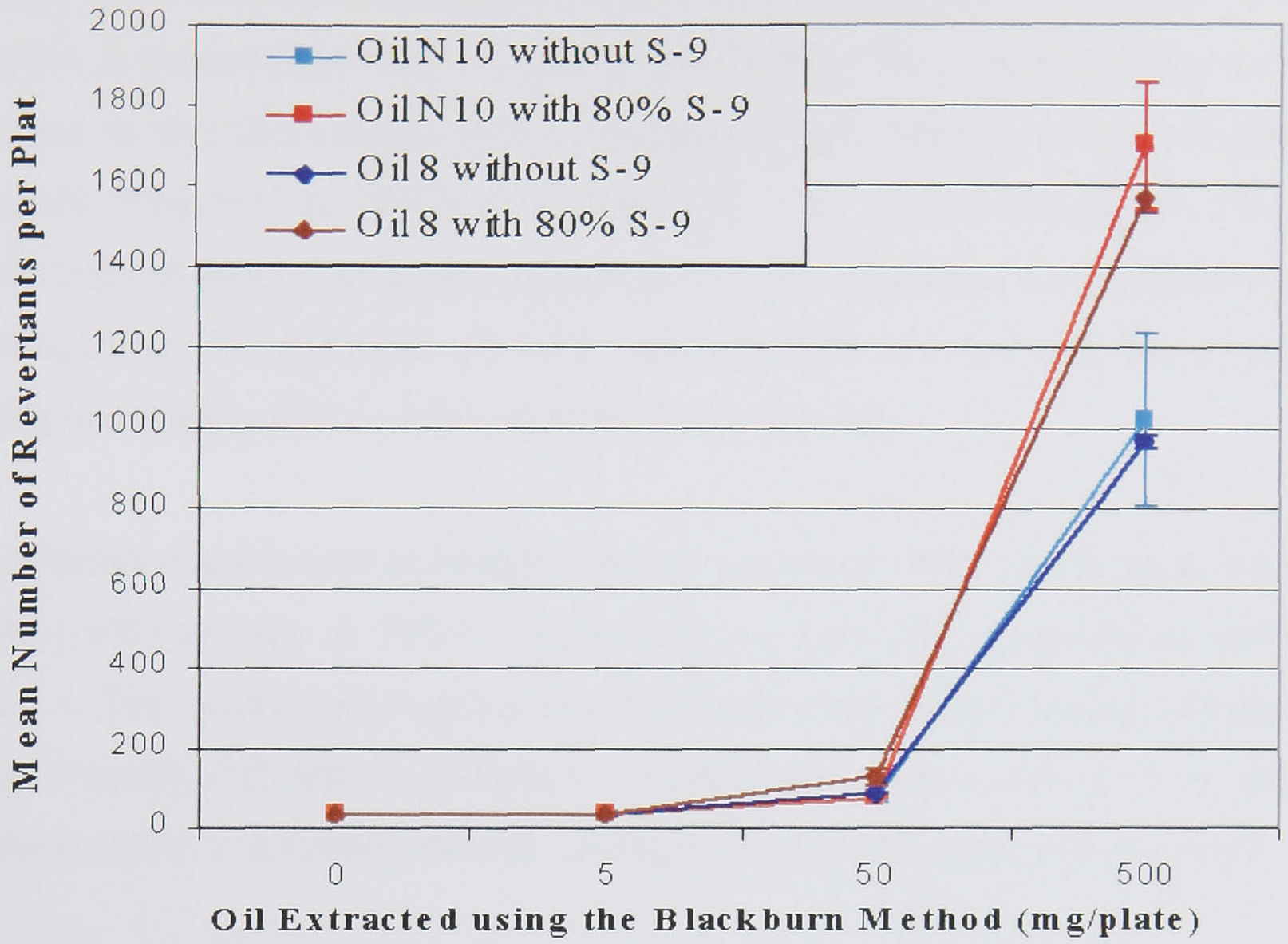


FIGURE 5.2. The Ames test revertants on the addition of Blackburn oil extracts of oil Nytro-10GBN and oil 8 over 4 doses in the absence and presence of S-9 (n = 3). %CV ranges from 2.4-21.0%.

5.3 MINISCREEN TEST FOR C18/SILICA/ISOLUTE PAH HC EXTRACTS

A number of LLE in the literature have been investigated for mutagenicity, but do not produce PAH specific extracts. The C18/Silica/Isolute PAH HC extracts must be tested for mutagenicity as the GC-MS data indicated it was an improved method of PAH isolation. In order to test the small volumes of extract recovered from the C18/Silica/Isolute PAH HC method however, a small scale mutagenicity test was required. The approach of the Miniscreen test is based on the Ames test, but uses smaller plates to allow a 5-fold reduction in the amount of sample required (Section 2.2.12).

This approach seemed promising as it required no extra training or expertise for Ames test users. A potential problem was that with a smaller area available for growth, the difference in revertant number on a mutagenic and non-mutagenic plate was difficult to discern. However, the literature suggested that this was not significant, providing that the test remained a screening method and was not treated as the ultimate measure of mutagenicity. Nevertheless, on using this approach it was found that it was not possible to make conclusive measurements of mutagenicity.

The Grimmer extracts used in Section 3.4 were used in the Miniscreen test as stated by Brooks (1995) initially. A 10% v/v of S-9 mix was used and the results are shown in Table 5.1. The positive and negative controls in particular showed that the results were not significantly different. Growth area was minimal thus less colonies could grow; it was not possible to determine toxicity due to the low spontaneous revertant count.

TABLE 5.1. Miniscreen results for transformer oil Grimmer extracts, Brooks *et al.*, (1995).

Miniscreen Plate with Grimmer extract (20 µgmL-1)	*Number of revertants*			*Mean Number of revertants*	*SD (n = 3)*	*%CV*
Negative Control	1	2	1	1	0.6	43
Positive Control	4	2	3	3	1.0	33
White Oil without S-9	1	3	1	2	1.2	69
White Oil with S-9	2	1	5	3	2.0	78
Oil 4 without S-9	2	4	5	4	1.5	42
Oil 4 with S-9	2	2	4	3	1.2	43
Oil N10 without S-9	5	3	6	5	1.5	33
Oil N10 with S-9	5	3	2	3	1.5	46
Oil 8 without S-9	4	2	5	4	1.5	42
Oil 8 with S-9	2	5	2	3	1.7	58

The same experiment was repeated with 10% S-9 using the modifications of Burke *et al.,* (1996) (Section 2.2.12). The revertant count quoted by the researchers for the positive control of *S. typhimurium* TA98 with 2-amino anthracene and S-9 was 15-182 and in the absence of S-9 was 0-16. The results in Table 5.2 proved no more conclusive than those obtained by the Brooks (1995) protocol. The modified method of Burke *et al.,* (1996) was repeated with Grimmer extracts with 80% S-9 but proved inconclusive as all plates were too opaque to count due to the high concentration of S-9. Since previous work showed that 80% v/v S-9 was required for detection, the Miniscreen was considered unsuitable for oil extract testing.

TABLE 5.2. Miniscreen results for the Grimmer extracts of transformer oils, Burke *et al.,* (1996).

Miniscreen Plate with Grimmer extract (20 µgmL-1)	*Number of revertants*			*Mean Number of revertants*	*SD (n = 3)*	*%CV*
Negative Control	3	2	3	3	0.5	22
Positive Control	6	8	9	8	1.5	20
White Oil without S-9	5	3	4	4	1.0	25
White Oil with S-9	7	3	5	5	2.0	40
Oil 4 without S-9	2	4	4	3	1.2	35
Oil 4 with S-9	5	4	5	5	0.6	12
Oil N10 without S-9	5	3	6	5	1.5	33
Oil N10 with S-9	5	4	5	5	0.6	12
Oil 8 without S-9	4	3	5	4	1.0	25
Oil 8 with S-9	8	8	6	7	1.2	16

As miniaturisation of the Ames test proved unsuccessful, scaling up of the extraction method was considered. As all other extraction methods had been tested using the standard Ames test, this allowed direct comparison of new data with previous results. However, the extracts were analysed first with GC-MS to determine how the large scale extraction differs from the small scale method.

5.4 CHANGES IN PAH CHROMATOGRAMS FOR LARGE SCALE C18/SILICA/ISOLUTE EXTRACTION

In order to perform a large scale C18/Silica/Isolute PAH HC extraction, C18 and silica (SiO_2) were purchased as commercially prepared 10g columns. The 1 g Isolute PAH HC columns were the only size available, thus all oil extracts were divided equally between 10 Isolute columns after extraction with C18 and SiO_2. Further details are given in Section 2.2.10.

The chromatogram for the large scale extractions are shown for oil 8 (Figure 5.3), Nytro-10GBN (Figure 5.4) and oil 4 (Figure 5.5). Each chromatogram was less complex than its Grimmer or IP 346 counterpart, which can be seen by comparing Figure 5.3 with Figure 3.7. However, the large scale C18/Silica/Isolute PAH HC chromatogram exhibited more interferent peaks than the small scale method. Oil 8 was still too complex and could not be used for identification of PAHs due to the presence of these interferents. Nytro-10GBN was less complex and could still be used to identify naturally occurring phenanthrenes eluted at retention times of >10 minutes, but the peaks were not as defined as those in the small scale extract (Figure 4.19). In addition there were many extra peaks at retention time 20-23 minutes which were not present in Figure 4.19. For this reason the small scale extracts were preferred for PAH identification in oil (Chapter 6).

The large scale extract for oil 4 showed no identifiable PAH peaks, which was not unexpected given that oil 4 had a relatively low PAH content. However, the chromatogram also exhibited less interfering peaks. This was also the case for white oil (Figure 5.6). This suggested that oil 8 was not only more complex in its PAH composition, but also in its general composition.

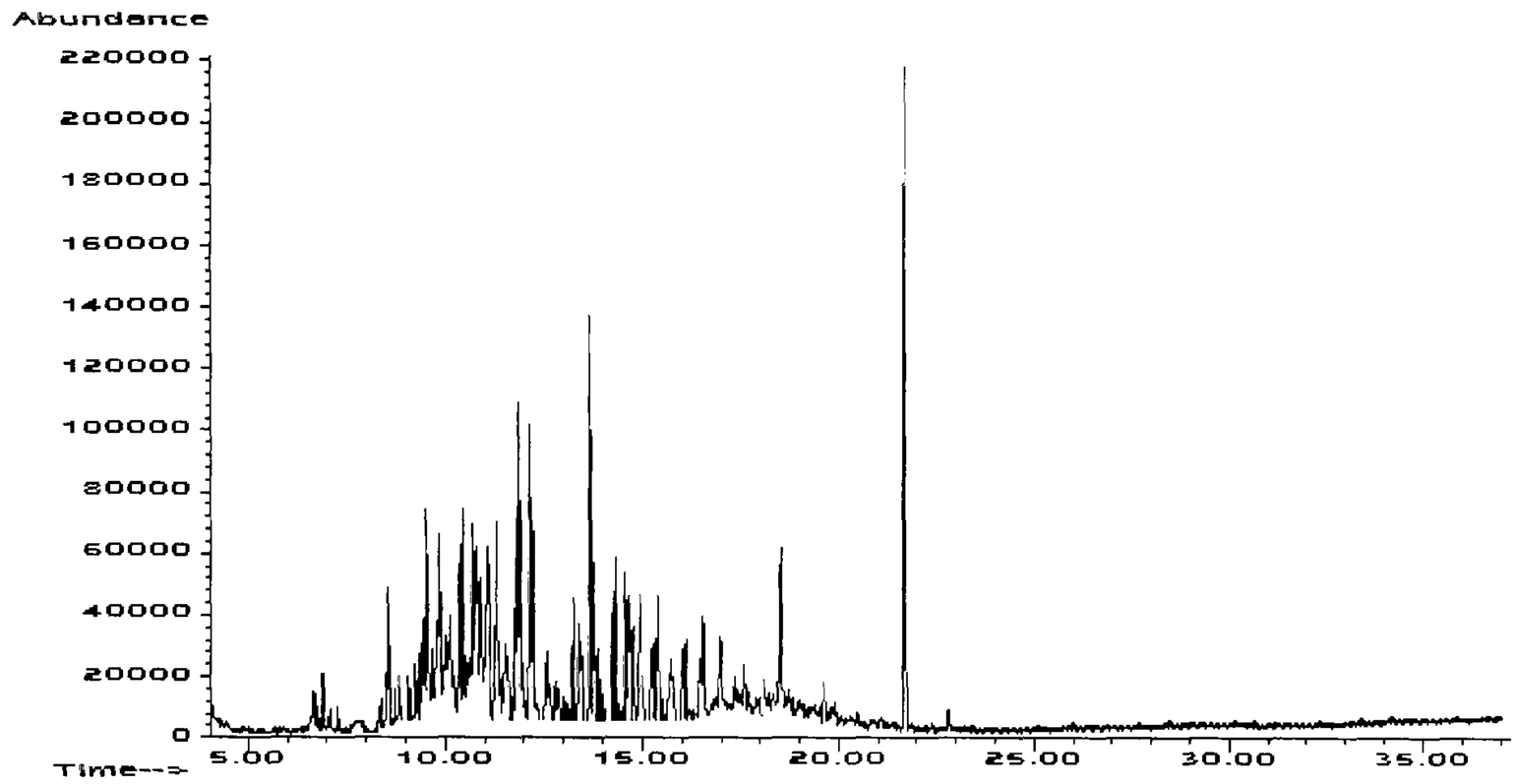


FIGURE 5.3. TIC of oil 8 large scale C18/silica/Isolute PAH HC extract.

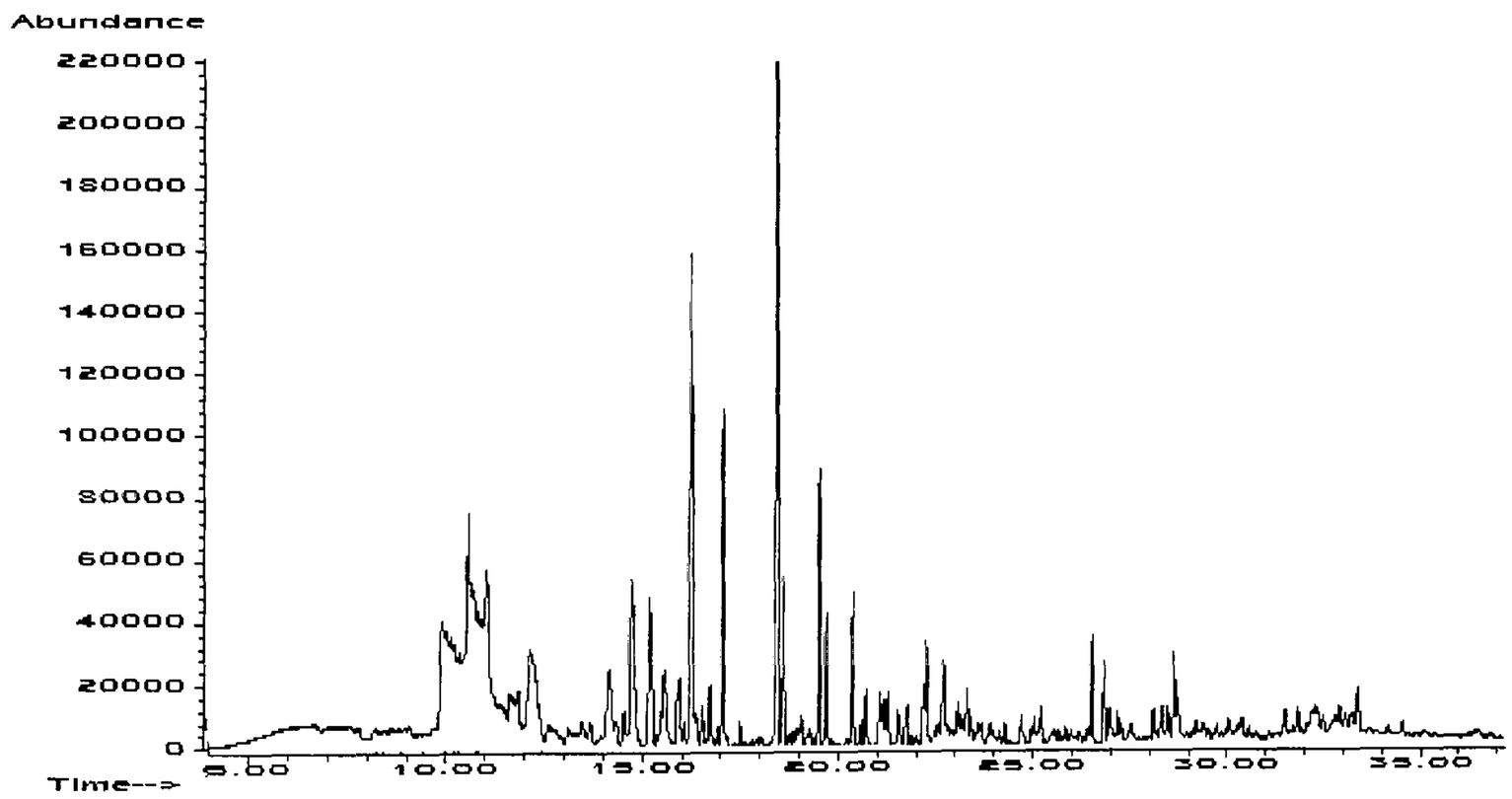


FIGURE 5.4. TIC of oil Nytro-10GBN large scale C18/silica/Isolute PAH HC extract.

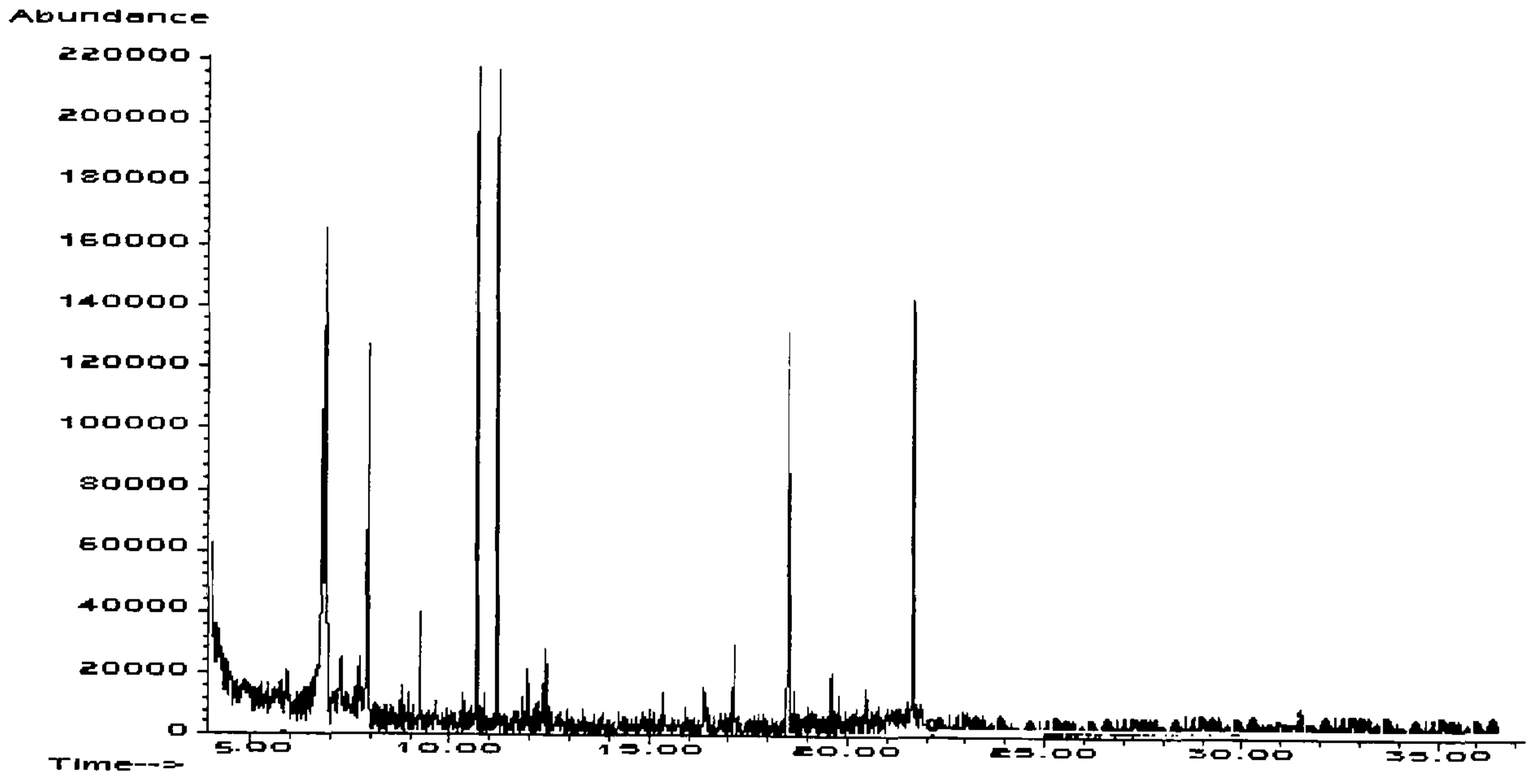


FIGURE 5.5. TIC of oil 4 large scale C18/silica/Isolute PAH HC extract.

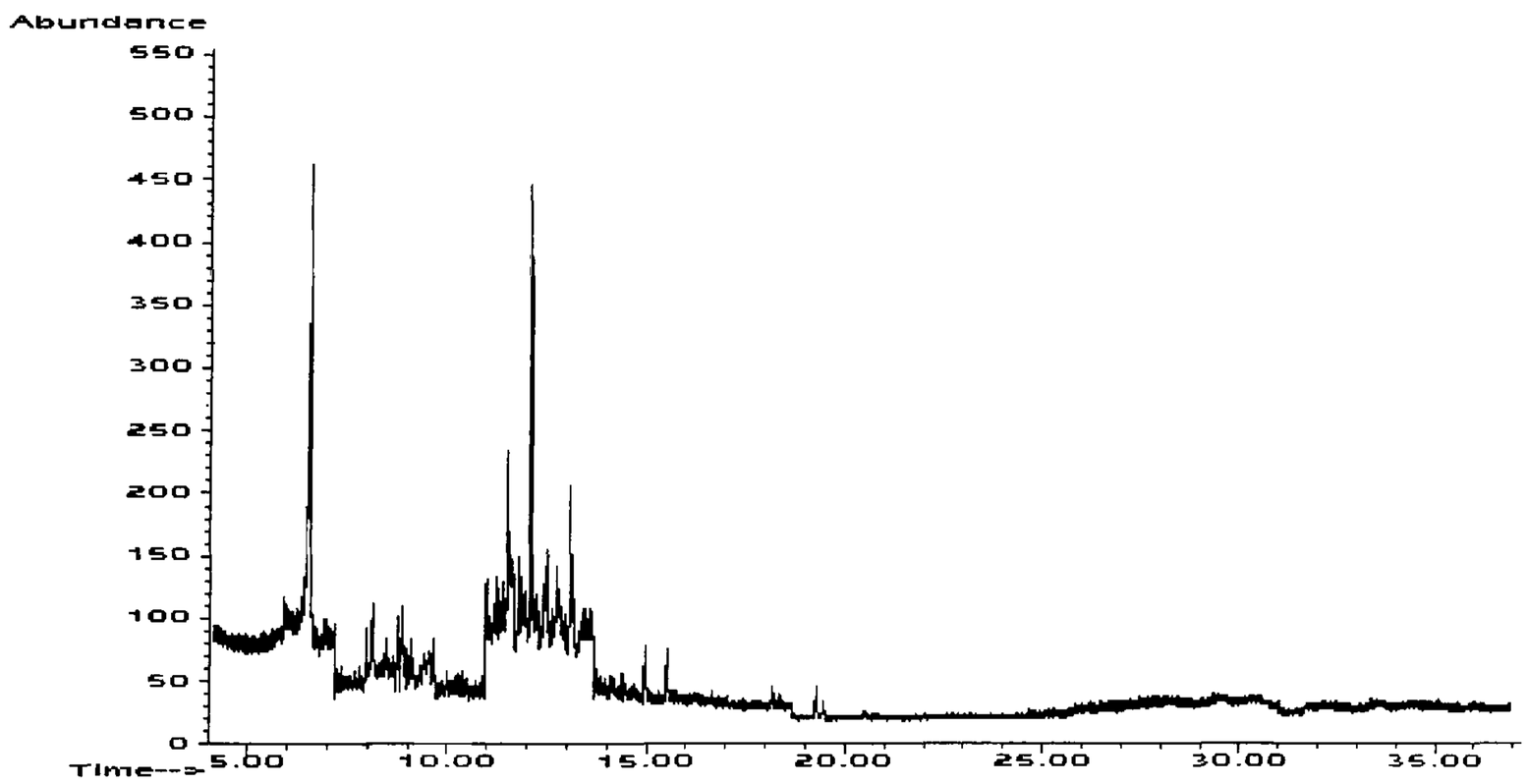


FIGURE 5.6. TIC of white oil large scale C18/silica/Isolute PAH HC extract.

As the large scale extraction method was less effective than the small scale method, a small scale extract was also tested for mutagenicity (Section 5.5.3) to determine if the additional interferents in the large scale extract affected Ames testing.

5.5 AMES TESTING OF C18/SILICA/ISOLUTE PAH HC EXTRACTS

5.5.1 Testing Column Residue for Interference

As seen in Section 4.3.3, the Isolute PAH HC column produced column bleed that affected the GC-MS chromatogram of transformer oils (retention time 21 minutes). With possible additional column bleed from the C18 and silica columns, it was imperative that the mutagenic properties from such column components were identified before C18/Silica/Isolute PAH HC extracts were tested for mutagenicity.

Table 5.3 shows the results of two large scale C18/Silica/Isolute PAH HC extractions performed without sample added. Two large scale extractions were run since two extract volumes were required to produce sufficient extract for testing (Section 2.2.10). It can be seen that the column residue did not produce a mutagenic response with or without 80% v/v S-9. The method therefore exhibited compatibility with the Ames test.

TABLE 5.3. Number of revertants on the addition of column residue from C18/silica clean up with Isolute PAH HC purification with and without 80% S-9.

Sample on Plate	*Number of revertants*			*Mean Number of revertants*	*± SD (n = 3)*	*%CV*
Negative Control (DMSO)	27	32	33	31	3	10
Positive Control (B[a]P)	251	265	281	266	15	6
Column Residue without S-9	29	34	32	32	3	8
Column Residue with 80%S-9	31	29	29	30	1	4

5.5.2 Mutagenicity of Oil with Large Scale C18/Silica/Isolute PAH HC Extraction with 80% S-9

Figure 5.7 shows the Ames test results for white oil and oil 4 using the large scale C18/Silica/Isolute PAH HC extracts. The revertant numbers of individual Ames test plates is given in Appendix C. No direct mutagenicity was observed for white oil and oil 4 suggesting that the direct mutagens present in both Grimmer and Blackburn extracts were not extracted by the C18/Silica/Isolute PAH HC method. This suggests that by measuring PAH content only, the full mutagenic nature of the oil is not observed. This further confirms that PAHs may not be the main source of unrefined oil mutagenicity as stated in the literature (Grimmer, 1983, McKee *et al.,* 1989; Järvholm and Easton, 1990; Brooks *et al.,* 1995) for these particular oils.

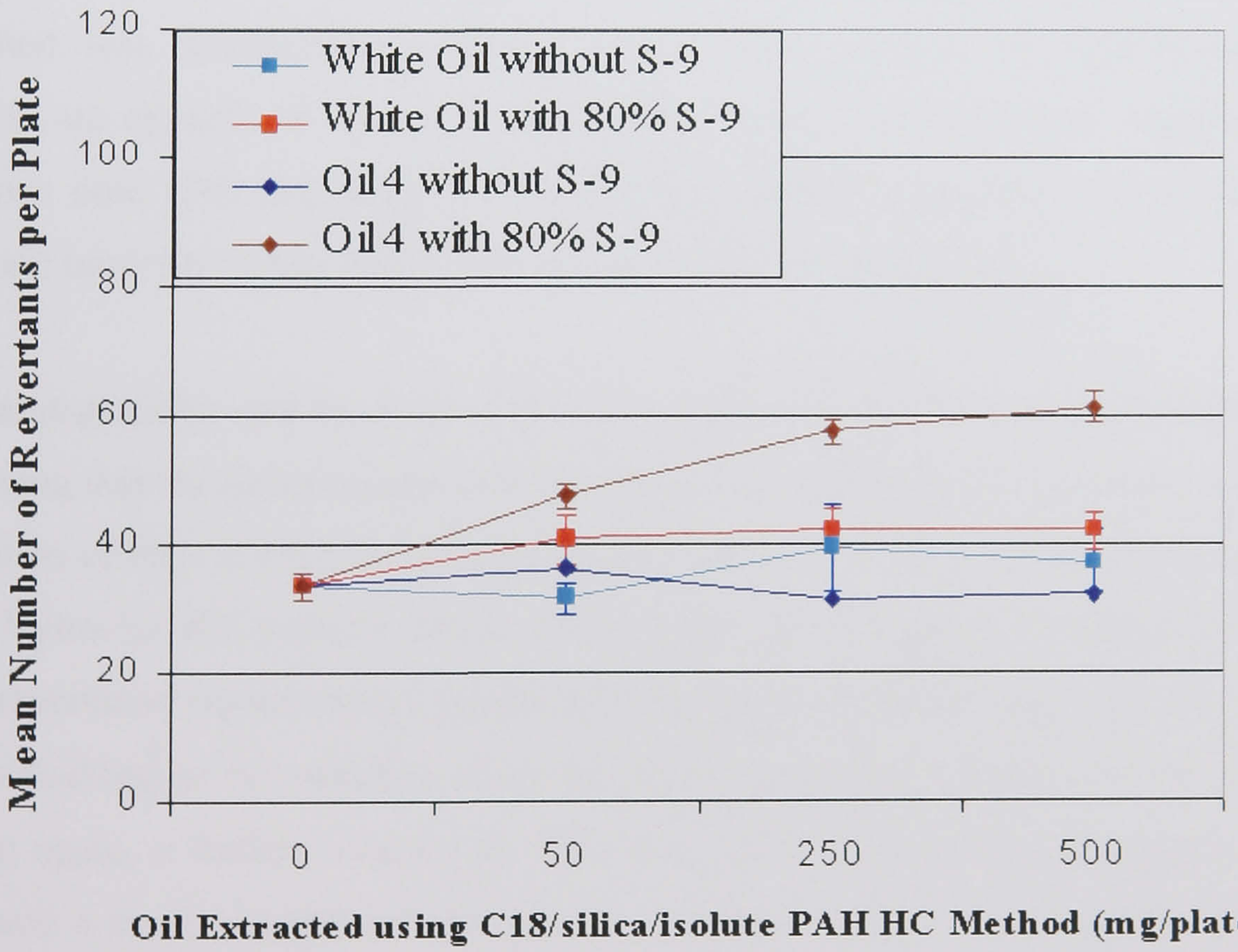


FIGURE 5.7. Mutagenicity of the C18/silica/Isolute PAH HC extract of white oil and oil 4 over 4 doses in the absence and presence of S-9 (n = 3). %CV ranges from 4.6-9.9%.

When S-9 was added, white oil continued to show no mutagenicity, and therefore correlated with the fact that white oil contains a low aromatic content. Oil 4 however, produced a 62 revertant increase at 500 mg plate^{-1}, on the addition of S-9 representing a near-doubling of the background spontaneous rate (33 revertants) but an exact doubling when compared to the number of revertants at the same dose without S-9 (31 revertants). This indirect mutagenicity was not observed with the Grimmer or Blackburn extracts. It was postulated that the presence of high levels of direct mutagens in the Grimmer and Blackburn extracts had obscured this indirect mutagenicity.

However, although there was a dose response observed, a further increase in dose was required to ensure the results were repeatable. Previous comparison of extract mutagenicity (Blackburn with Grimmer extracts) was sufficient to validate a response observed at only one dose. However, as the C18/Silica/Isolute PAH HC extraction method was substantially different to the LLE methods of both Grimmer and Blackburn extractions, it was postulated that further validation was required. As the highest dose (500 mg plate^{-1}) of extract was partially saturating the DMSO carrier solvent however, it was difficult to increase the dose substantially.

No mutagenicity was observed with oil 8 and Nytro-10GBN without S-9 (Figure 5.8) showing that the direct mutagens were absent as in the white oil and oil 4 extracts. The addition of 80% v/v S-9 however, did increase the number of revertants for both oil 8 and Nytro-10GBN extracts. Nytro-10GBN, like oil 4 showed an intermediate response (71 revertants) representing a doubling of the spontaneous reversion number (33) but a near-doubling of the response observed at the same dose, without S-9 (38 revertants). Once again, a further increase in dose was required for testing mutagenicity. Oil 8 showed a more pronounced increase in revertant number (~100) on the addition of 80% v/v S-9; clearly a mutagenic response. Even this significant response however, required qualification with further measurement at a higher dose, to determine if the mutagenic response was still present.

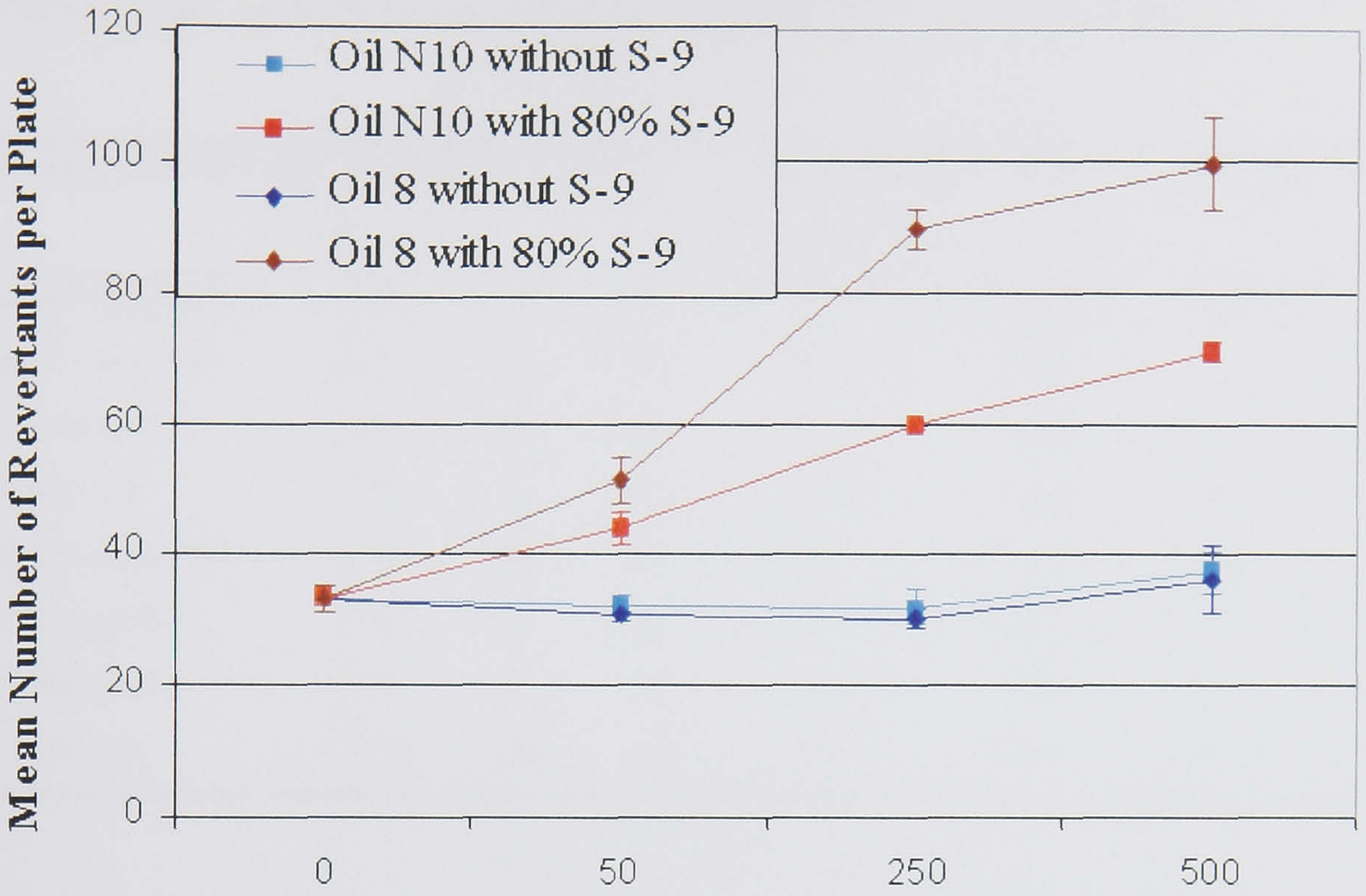


FIGURE 5.8. Mutagenicity of the C18/silica/Isolute PAH HC extract of oil Nytro-10GBN and oil 8 over 4 doses in the absence and presence of S-9 (n = 3). %CV ranges from 3.2-6.3%.

Due to solvent saturation issues, a final dose of 580 mg plate^{-1} (an increase of 16%) was achieved as the highest dose available for testing in a minimal solvent volume of 0.6 mL DMSO. The results are shown in Table 5.4 and indicate the same findings as those obtained at 500 mg plate^{-1}, concluding that the mutagenic response observed for oil 4, 8 and Nytro-10GBN with 80% v/v S-9 was reproducible and therefore significant. Mutagenicity of the C18/Silica/Isolute PAH HC extracts compared to the Grimmer and Blackburn extracts is further discussed in Section 7.4.2.1.

TABLE 5.4. Number of revertants on the addition of Nytro-10GBN extracted by C18/silica clean up with Isolute PAH HC purification at 580 mg plate^{-1}.

Sample on Plate (580 mg)	*Number of revertants*			*Mean Number of revertants*	*± SD (n = 3)*	*%CV*
White Oil without S-9	32	35	39	35	3.5	10
White Oil with S-9	35	39	37	37	2.0	5
Oil 4 without S-9	38	39	34	37	2.6	7
Oil 4 with S-9	79	72	75	75	3.5	5
Oil N10 without S-9	35	39	35	36	2.3	6
Oil N10 with S-9	79	84	80	81	2.6	3
Oil 8 without S-9	38	37	34	36	2.0	6
Oil 8 with S-9	115	124	116	118	4.9	4

5.5.3 Mutagenicity of Nytro-10gbn with Small Scale C18/Silica/Isolute PAH HC Extraction with 80% S-9

Results showed that the large scale extraction with C18/Silica/Isolute PAH HC PAH HC was not as effective at producing a clean PAH extract as the small scale version (Section 5.4). For this reason, small scale extracts were pooled and tested using the Ames test to determine if the large scale extract contained extra interference that altered mutagenicity. Nytro-10GBN was extracted using the small scale columns 20 times.

A volume of 330 µL oil was run down each column (instead of 100 µL used for GC-MS analysis) to reduce the number of extractions required. A volume of 400 µL of oil spiked with 1 µg mL^{-1} had previously been extracted in triplicate to ensure that the greater volume of oil did not affect extraction efficiency, which it did not. A 330 µL Nytro-10GBN oil sample produced an overall extract that was equivalent to the quantity of oil (5 g) extracted with the large scale version. This was not done for all the oils as it was both expensive and time consuming. Table 5.5 shows the results gained for small scale extraction at 500 mg plate^{-1}. The test was performed with the same *S.*

typhimurium culture used to test the large scale Nytro-10GBN extract, so results could be directly compared. Reversion number in Table 5.5 was approximately the same as that for large scale Nytro-10GBN extraction (Figure 5.8) at 500 mg plate^{-1}, suggesting that the presence of the extra interference in the large scale extract did not significantly affect mutagenicity results. The next stage was to determine if this cleaner extraction method altered the mutagenicity previously observed with aged oils.

TABLE 5.5. Numbers of revertants on the addition of Nytro-10GBN extracted by 20 x small scale columns with C18/silica clean up with Isolute PAH HC purification. Total oil extracted was 5 g.

Sample on Plate (500 mg)	*Number of revertants*			*Mean Number of revertants*	*± SD (n = 3)*	*%CV*
Negative Control	27	32	33	31	3.2	10
Positive Control (B[a]p)	289	248	286	274	22.8	8
Nytro-10GBN without S-9	35	34	32	34	1.5	5
Nytro-10GBN with S-9	69	78	76	74	4.7	6

5.6 AMES TESTING OF BLACKBURN AND C18/SILICA/ISOLUTE PAH HC EXTRACTS OF AGED OILS

5.6.1 Blackburn Extracts of Aged Oils with 80% S-9

Aged oils were extracted and tested to give an indication of how mutagenicity changes with age. PAHs are believed to increase with ageing (Moret and Conte, 2000; Wang *et al.*, 2000; Wong and Wang, 2001) and this could increase the risk of mutagenicity from oils. Such risks would increase the requirement for a full transformer oil service history and cause an increase in oil use, as the working life of an oil is reduced. The oil was extracted with the Blackburn method and tested before ageing (ALT 0) and after ageing for 1 week (ALT 1), 2 weeks (ALT 2) and 3 weeks (ALT 3). Results are reported in the absence (Figure 5.9) and presence of 80% v/v S-9 (Figure 5.10).

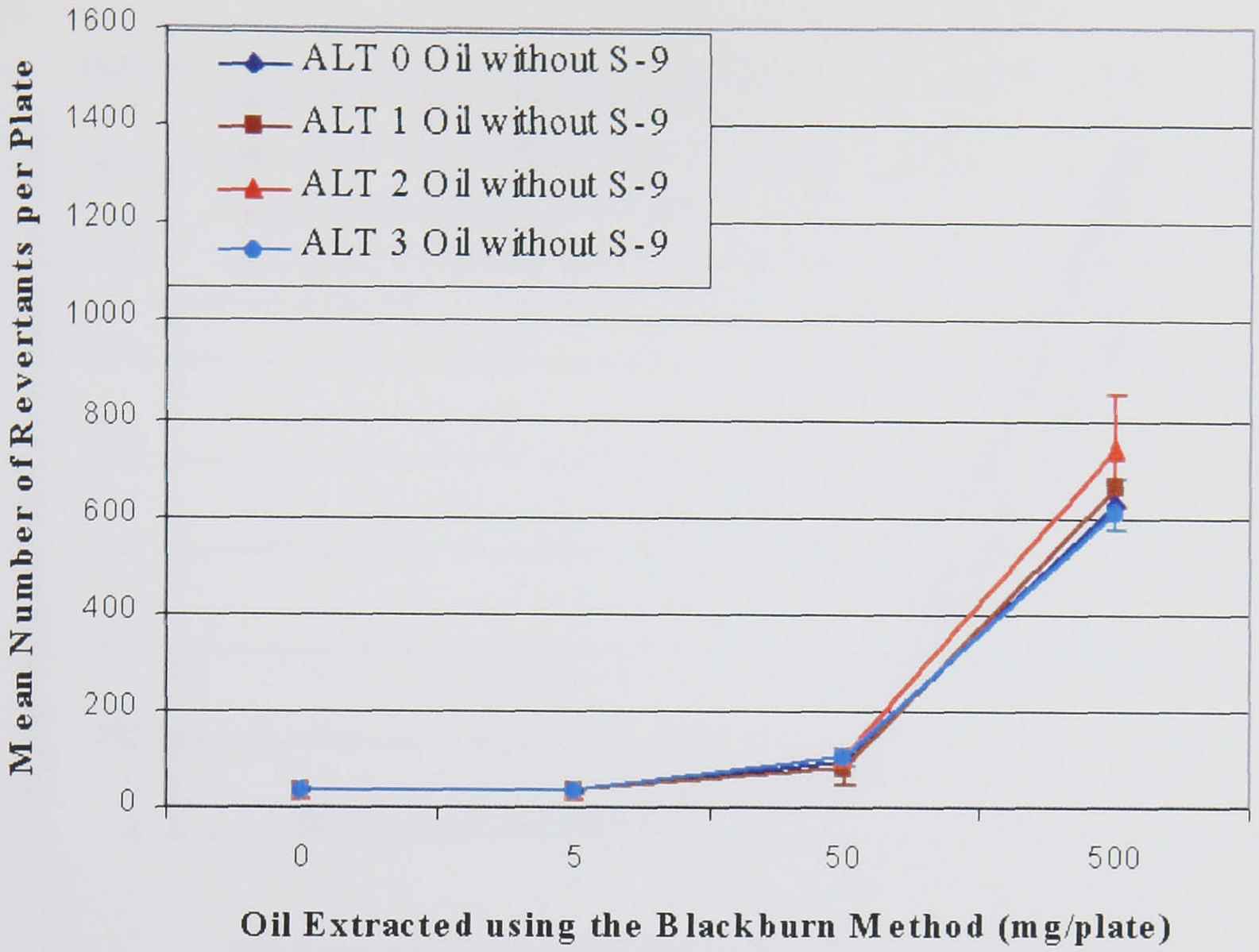


FIGURE 5.9. The Ames test revertants on the addition of Blackburn oil extracts of new oil ALT 0 and after ageing for 1 week (ALT 1), 2 weeks (ALT 2) and 3 weeks (ALT 3) without S-9 over 4 doses (n = 3). %CV ranges from 2.2-10.1%.

The results showed that Blackburn extracts of all oils were mutagenic with or without S-9. The number of revertants for each age of oil was not significantly different. It was therefore concluded that mutagenicity did not change with artificial ageing of up to 3 weeks. For the Blackburn extracts, revertant numbers increased from 550-850 revertants for all oils without S-9, to 1000-1600 revertants with 80% v/v S-9 at 500 mg plate^{-1}. This was approximately a doubling in revertant number, therefore suggesting that additional mutagenicity in the presence of 80% v/v S-9 was due to indirect mutagens such as PAHs. Indirect mutagenicity observed at only one dose was validated by comparison with the Grimmer extracts. The results correlate with the Grimmer extract results in Section 3.5 although the difference in revertant number with and without S-9 was greater for the Blackburn extracts and therefore more conclusive with regard to indirect mutagens.

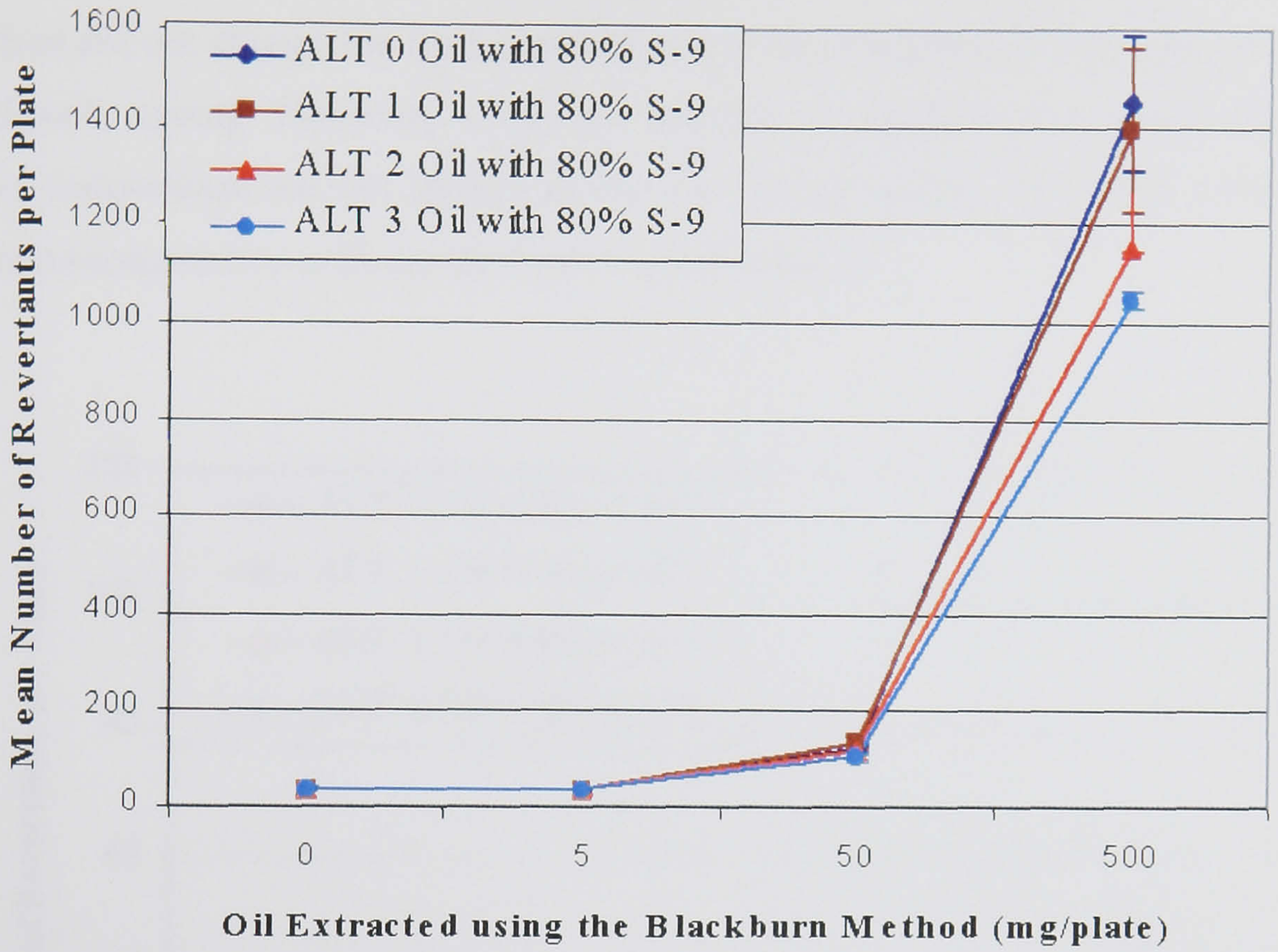


FIGURE 5.10. The Ames test revertants on the addition of Blackburn oil extracts of new oil ALT 0 and after ageing for 1 week (ALT 1), 2 weeks (ALT 2) and 3 weeks (ALT 3) with 80% S-9 over 4 doses (n = 3). %CV ranges from 4.0-11.9%.

5.6.2 C18/Silica/Isolute PAH HC Extracts of Aged Oil with 80% S-9

The results in Figure 5.11 showed that C18/Silica/Isolute PAH HC extracts of all aged oils contained no direct mutagens. This again indicated that the C18/Silica/Isolute PAH HC method did not extract significant quantities of direct mutagens as was the case with the Grimmer and Blackburn extracts (Section 3.4 and 5.2).

The results observed with 80% v/v S-9 (Figure 5.12) indicated mutagenicity for all stages of oil ageing but were not significantly different. These results indicated that mutagenicity did not change with artificial ageing of up to 3 weeks. This correlated with the data collected for both Grimmer and Blackburn extracts, although the

mutagenicity with S-9 was easier to differentiate with C18/Silica/Isolute PAH HC extracts as there was no direct mutagenicity. Therefore using an improved extraction method did not change the final conclusion that oil mutagenicity does not change with artificially ageing. However, it was not possible to conclude from these results that PAH composition did not change in the oils during ageing. This issue affecting oil extract mutagenicity is discussed further in Section 6.6.

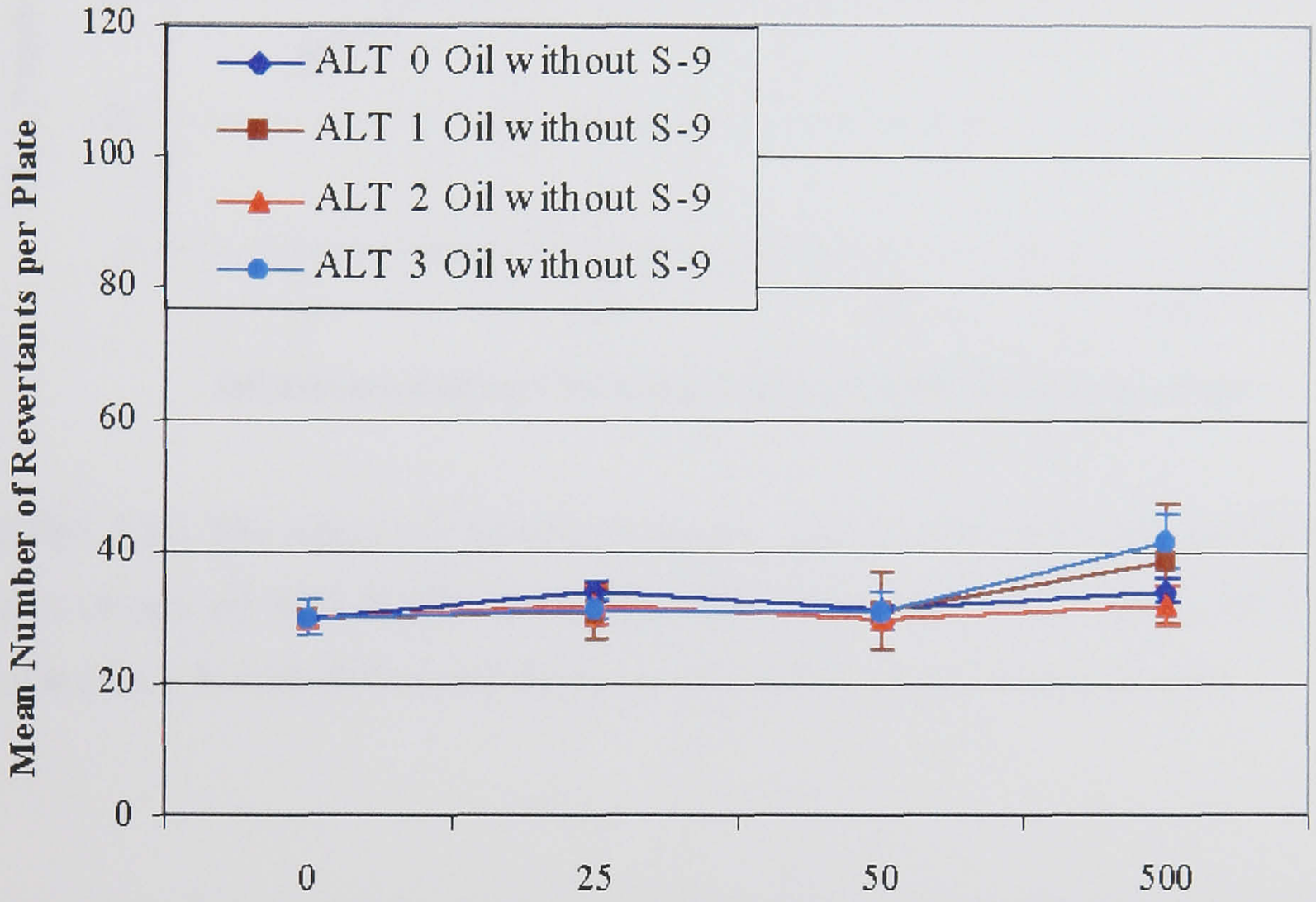


FIGURE 5.11. The Ames test revertants on the addition of C18/silica/Isolute PAH HC extracts of new oil ALT 0 and after ageing for 1 week (ALT 1), 2 weeks (ALT 2) and 3 weeks (ALT 3) without S-9 over 4 doses (n = 3). %CV ranges from 1.8-10.5%.

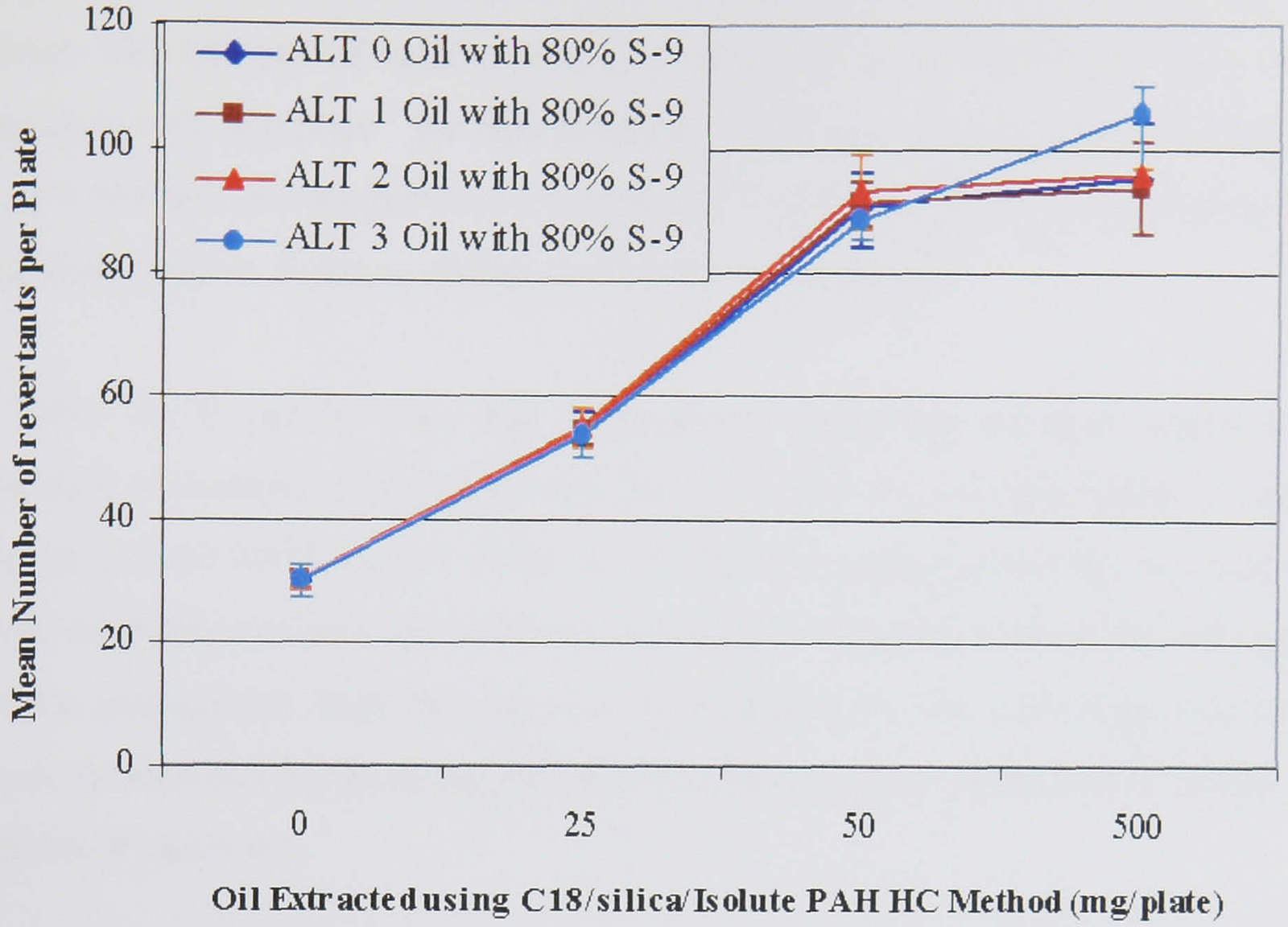


FIGURE 5.12. The Ames test revertants on the addition of C18/silica/Isolute PAH HC extracts of new oil ALT 0 and after ageing for 1 week (ALT 1), 2 weeks (ALT 2) and 3 weeks (ALT 3) with S-9 over 4 doses (n = 3). %CV ranges from 1.0-9.9%.

5.7 INVESTIGATION OF OIL EXTRACT INTERFERENCE ON THE AMES TEST

Although the C18/Silica/Isolute PAH HC extraction was more successful in reducing oil interferents than the Grimmer or Blackburn extracts, it was possible that the extract may still cause inhibition. The use of a less efficient large scale version of the C18/Silica/Isolute PAH HC extraction did not alter the mutagenicity measured with the small scale version (Section 5.5.3) but neither method produced a completely clean extract. For this reason, the inhibition effects of the C18/Silica/Isolute PAH HC extracts on individual PAH was tested according to the methods described in Section 3.8. The Blackburn extracts were also tested for comparison purposes.

Benzo[a]pyrene or 2-amino anthracene were added to the Ames test plates with oil extract. The amount of each extract (25 mg plate^{-1} for C18/Silica/Isolute PAH HC extracts and 50 mg plate^{-1} for Blackburn extracts) was used as it was the highest dose of oil tested that did not produce a mutagenic response, so only the mutagenicity of the benzo[a]pyrene or 2-amino anthracene would be observed.

In Table 5.6 it can be seen that none of the Blackburn extracts caused a greater inhibition of mutagen (32%) than non-extracted oil 8. As with the whole oil, inhibition increased as the PAH content of the oil increased, suggesting that the presence of other PAHs (possibly non-mutagenic PAHs) interfered with activation by inhibiting the S-9 enzyme preparation. This finding was strengthened by the t-test values in Table 5.6 which showed that the presence of white oil did not cause significant inhibition, whilst both oils 4 and 8 did.

TABLE 5.6. Effects on revertant number of adding Blackburn extracts to mutagens benzo[a]pyrene and 2-amino anthracene (10% S-9).

Sample on Plate	*Mean Number of revertants*	*SD (n = 3)*	*%CV*	*Reduction in Revertants on Exposure to Oil (%)*	*One-Sided t-Test (t) **
Benzo[a]pyrene (B[a]P)	196	34	17	N/A	N/A
Blackburn extract White oil and B[a]P	181	17	9	7	-1.4
Blackburn extract Oil 4 and B[a]P	168	11	6	14	-4.5
Blackburn extract Oil 8 and B[a]P	132	13	10	32	-8.1
2-amino anthracene (2-aa)	729	37	5	N/A	N/A
Blackburn extract White oil and 2-aa	708	19	2	3	-1.9
Blackburn extract Oil 4 and 2-aa	649	25	4	11	-5.5
Blackburn extract Oil 8 and 2-aa	564	20	3	23	-15

**Critical Value t3 = -2.35 (P = 0.05)*

The same trend is seen in Figure 5.7 for the C18/Silica/Isolute extracts. However, in this case inhibition did affect the results measured in the Ames test, as both oil 4 and Nytro-10GBN gave less significant results (Section 5.5.2). A 15% increase in revertant number for oil 4 would have strengthened the indicated mutagenicity. This showed that the C18/Silica/Isolute PAH HC extract, even though cleaner and with lower levels of direct mutagens in the oil, still contained a matrix of sufficient complexity to alter PAH mutagenicity. For this reason, inhibition was looked at in more detail.

TABLE 5.7. Effects on revertant number of adding C18/silica/Isolute PAH HC extracts to mutagens benzo[a]pyrene and 2-amino anthracene (10% S-9).

Sample on Plate	*Mean Number of revertants*	*SD (n= 3)*	*%CV*	*Reduction in Revertants from Mutagen on Exposure to Oil (%)*	*One-Sided t-Test (t) **
Benzo[a]pyrene (B[a]P)	196	13	7	N/A	N/A
C18/Silica/Isolute PAH HC extract White oil and B[a]P	173	31	18	12	-1.2
C18/Silica/Isolute PAH HC extract Oil 4 and B[a]P	166	32	19	15	-1.5
C18/Silica/Isolute PAH HC extract Oil 8 and B[a]P	136	7	5	30	-15
2-amino anthracene (2-aa)	729	37	5	N/A	N/A
C18/Silica/Isolute PAH HC extract White oil and 2-aa	653	33	5	10	-4.0
C18/Silica/Isolute PAH HC extract Oil 4 and 2-aa	616	30	5	15	-6.4
C18/Silica/Isolute PAH HC extract Oil 8 and 2-aa	536	27	5	26	-12

**Critical Value t3 = -2.35 (P = 0.05)*

5.7.1 Inhibition at 80% S-9

The previous inhibition tests (Section 3.8 and 5.7) were performed with 10% v/v S-9. Increasing the S-9 concentration would provide more enzymes for mutagen activation and would determine if the presence of PAHs was affecting the activation of the mutagen. If PAHs in the oil extracts were binding to the enzyme, this effect would decrease with increasing S-9 concentration. This was shown to be true (Table 5.8). The reduction in the number of revertants was much smaller with the addition of 80% v/v S-9, suggesting that the PAH content of oil was interfering with the activation of mutagenic PAHs. The inhibition of oil 4 and Nytro-10GBN at 80% v/v S-9 was not sufficient to alter the Ames test results for C18/Silica/Isolute PAH HC extracts in section 5.5.2. However, even with 80% v/v S-9, (according to the t-test) oil 8 significantly reduced the mutagenicity of 2-amino anthracene, as expected, given that oil 8 had a large aromatic content. Inhibition is further discussed in Section 7.5.1.

TABLE 5.8. Effects on revertant number of adding C18/silica/Isolute PAH HC extracts to mutagen 2-amino anthracene (80% S-9).

Sample on Plate	*Mean Number of revertants*	*SD (n = 3)*	*%CV*	*Reduction in Revertants from Mutagen on Exposure to Oil (%)*	*One-Sided t-Test (t) **
2-amino anthracene (2-aa)	909	16	2	N/A	N/A
C18/Silica/Isolute PAH HC extract White oil and 2-aa	903	38	4	0.6	-0.2
C18/Silica/Isolute PAH HC extract Oil 4 and 2-aa	896	31	3	1.4	-0.7
C18/Silica/Isolute PAH HC extract Oil 8 and 2-aa	885	15	2	3	-2.7

**Critical Value t3 = -2.35 (P = 0.05)*

Although inhibition effects were shown to be insignificant for the extractions performed in this work, there was another possible interference issue that required investigation to fully explore the mutagenic potential of PAHs in oil. It was possible that toxic effects, first observed in Section 3.2.1.2, could be affecting the mutagenicity test to the extent that mutagenicity is no longer observed. Toxicity testing also brought into question other potential hazards associated with oil, which should be addressed.

5.8 TOXICITY OF OIL EXTRACTS

Many methods for measuring the toxicity of a sample are available. However, for the purpose of this work, a simple toxicity test, using *S. typhimurium* was developed. *S. typhimurium* is not an ideal organism for determining the hazard to human health, as it is a prokaryote. However, the primary concern of this work was the investigation of toxicity on the Ames test (Section 1.3.2). Toxicity of a test sample would lead to a reduction in the number of viable *S. typhimurium* cells. With fewer cells to revert, the number of colonies counted on an Ames test plate would be reduced and may lead to false negative results. *S. typhimurium* was therefore the appropriate choice of test organism.

The method used for toxicity testing is outlined in Section 2.2.11. Absorbance measurements were performed in a microtitre plate to allow high sample throughput. The wavelength that gave the greatest absorbance reading with *S. typhimurium* growth in nutrient broth was 600 nm.

The top agar of the Ames test was ~4% v/v *S. typhimurium* culture and ~4% sample, so toxicity testing was performed by reproducing relative quantities (Table 5.9). As the microwell plates are x10 smaller than the Ames test plates, the toxicity observed at 50 mg well^{-1} is equivalent to the highest dose (500 mg plate^{-1}) of the Ames test (both equate to the same final concentration). A measure at a dose of 75 mg well^{-1} was performed to determine toxic effects at a level greater than that tested by the Ames test. As previously discussed (Section 1.4.1.6) many extract had solubility issues with respect to the carrier solvent at higher doses. The dose of 75 mg well^{-1} therefore, was

difficult to achieve, without vortexing of the sample (highest speed available) before use. This test was not directly comparable with the Ames test as it used a different media and a different measurement parameter, but it gave an indication of possible toxicity issues in the Ames test.

TABLE 5.9. The volumes of components in the *S. typhimurium* toxicity test compared to the Ames test.

Component added	*Ames Test (µL)*	*Toxicity Test (µL)*
S. typhimurium	100	10
Media	2500*	250
Sample	100	10

** Refers to the top agar with S-9 volume included*

An important factor in testing for toxicity was determining what reduction in absorbance would be considered significant. A reduction in growth (absorbance) of 10% and particularly 50% (LD_{50}) are often measured as and indication of toxicity (Toxicological Research Centre, 2003; ISO 10712, 1995). As this toxicity test has not been performed before and therefore has not been standardised or optimised, there may be a level of imprecision. From repeating the test 10 times with a sample of Nytro-10GBN Grimmer extract a %CV of up to 10% was observed. For this reason a >20% reduction in growth was used as an indicator of inhibition instead of 10%. Results were therefore expressed in terms of their relation to a 20% and 50% decrease in the absorbance observed at 0 mg $well^{-1}$.

5.8.1 Toxicity of Oil 8, 4, Nytro-10GBN and White Oil Extracts

Results for the Grimmer extracts of oil 8, Nytro-10GBN, 4 and white oil are shown in Figure 5.13. At 50 mg well^{-1} no absorbance drop of 20% or greater was observed. However, both oil 8 and Nytro-10GBN showed a decline in absorbance of >20% at 75 mg well^{-1}. This suggested that extending the mutagenicity measurement to a higher dose would have been subject to toxicity effects.

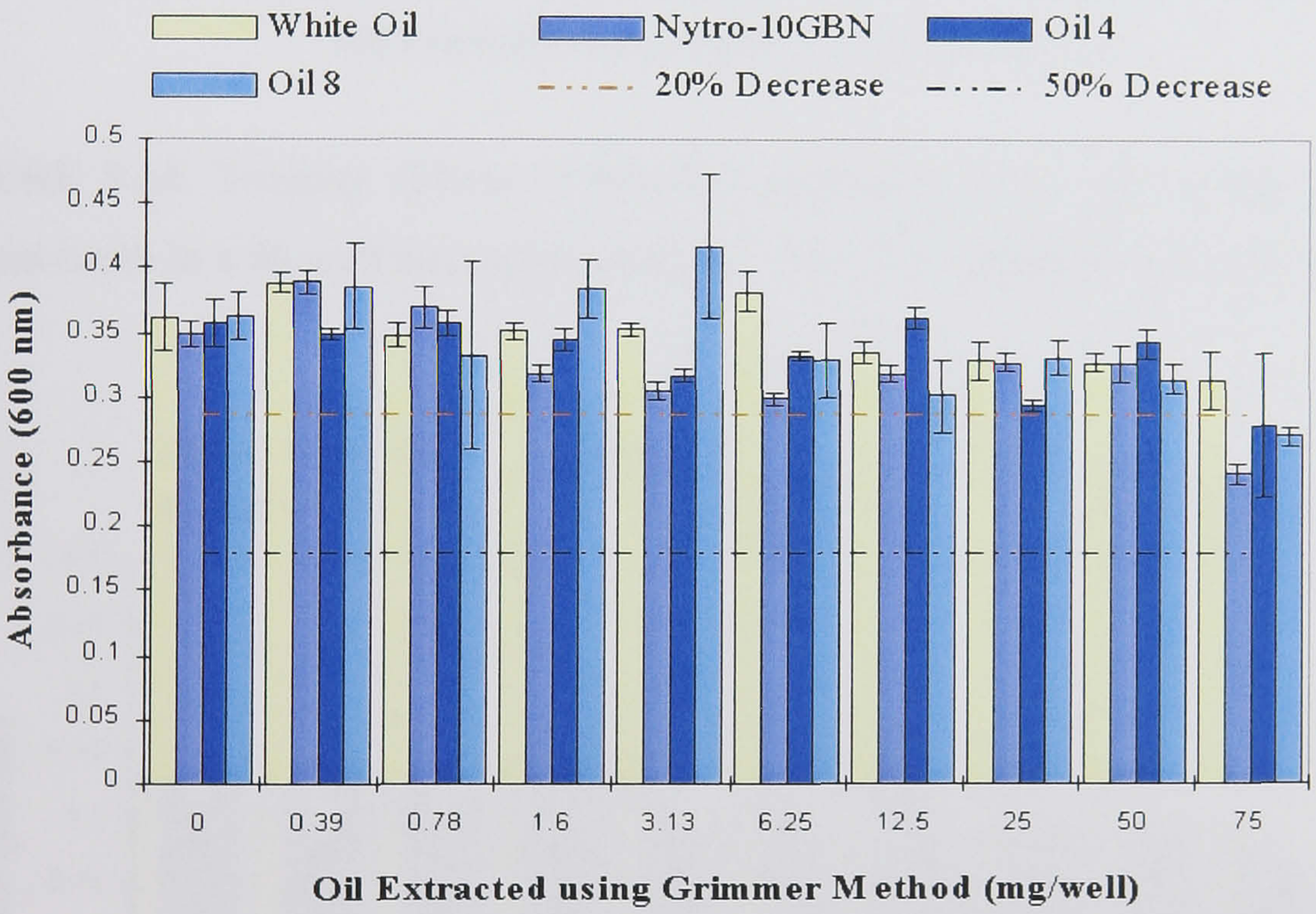


FIGURE 5.13. Toxicity effects of Grimmer extracts on *S. typhimurium* grown in nutrient broth in a 96 well microtitre plate (n = 3). %CV ranges from 0.4-21.7%.

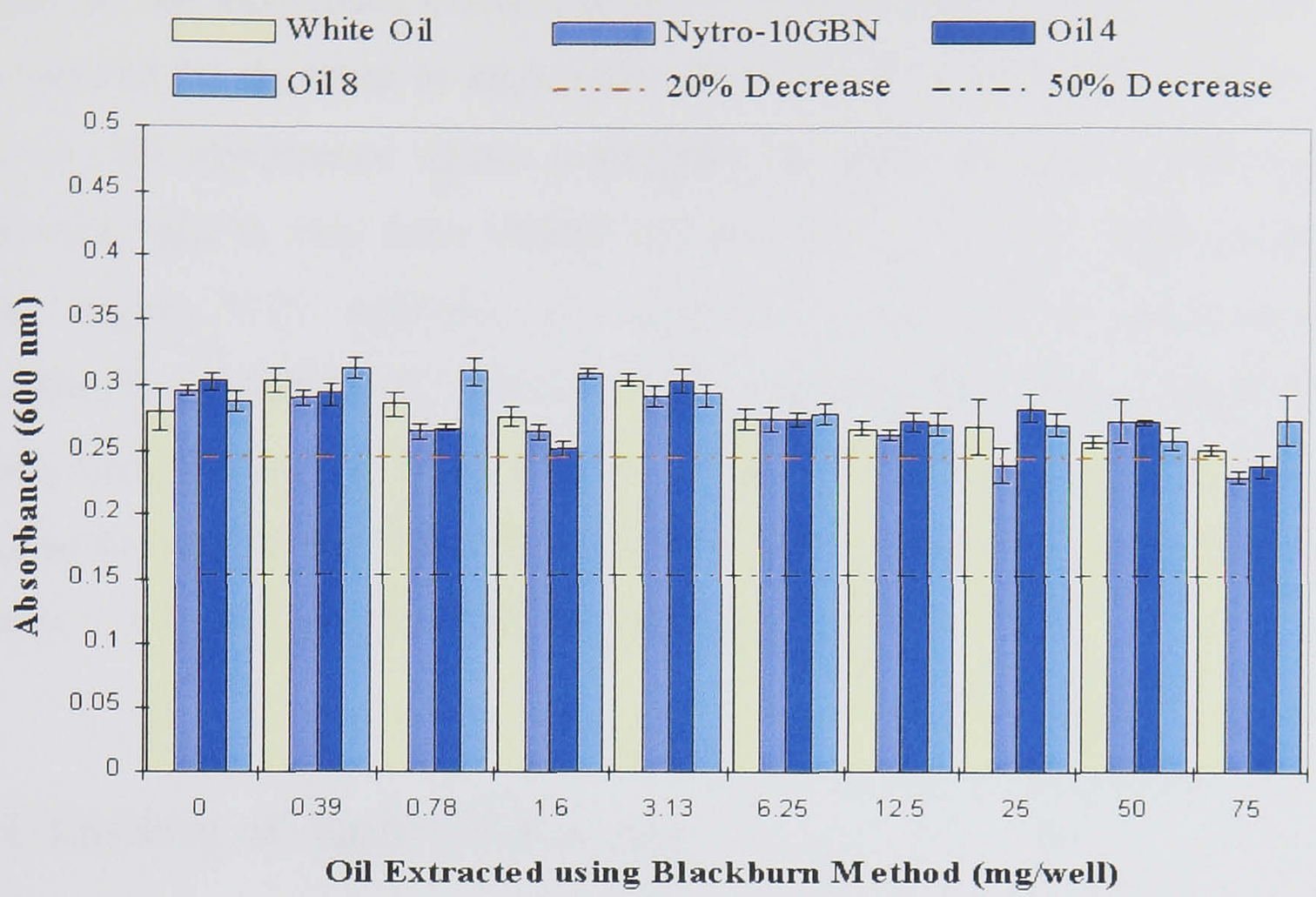


FIGURE 5.14. Toxicity effects of Blackburn extracts on *S. typhimurium* grown in nutrient broth in a 96 well microtitre plate (n = 3). %CV ranges from 0.5-7.7%.

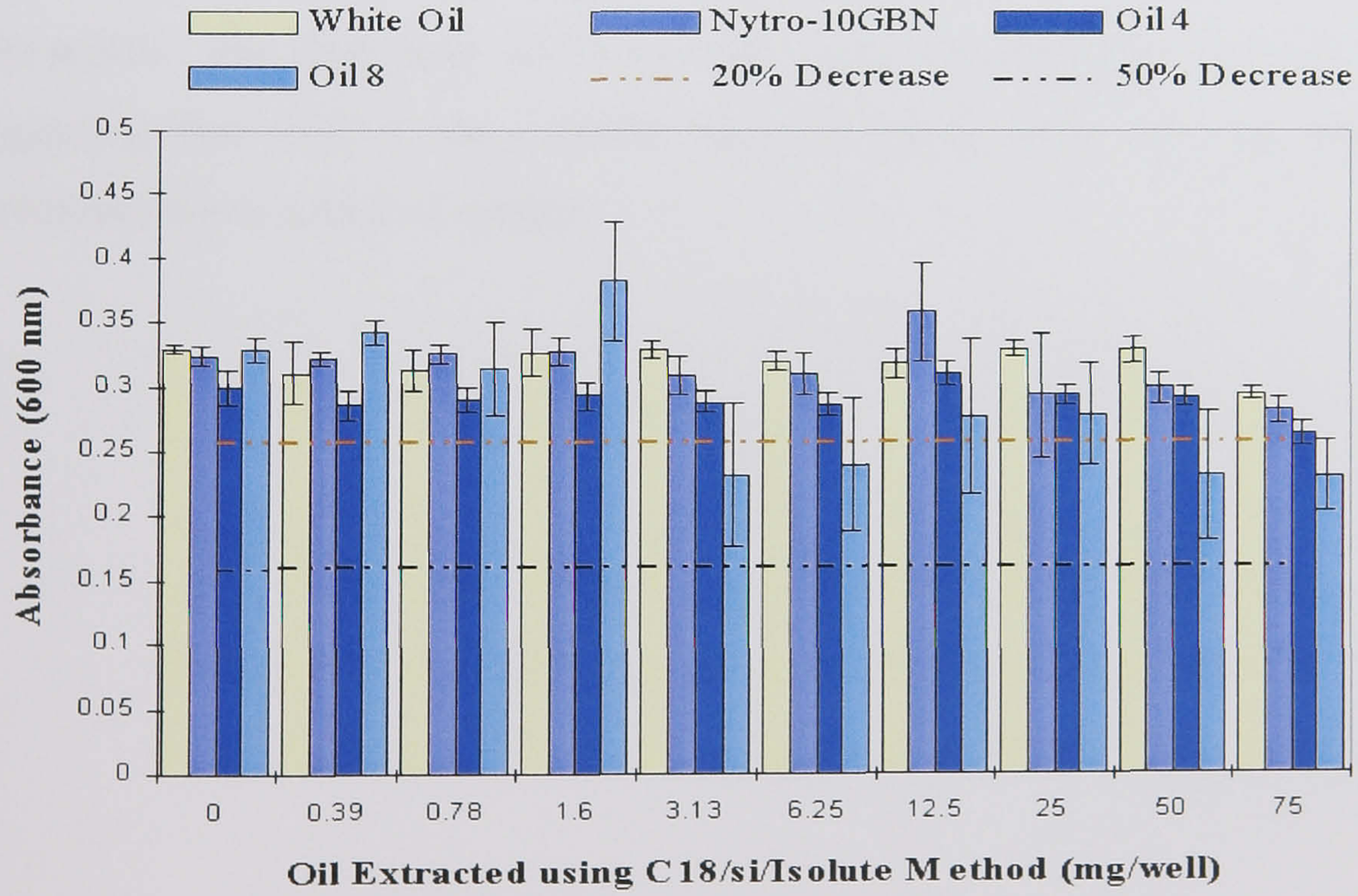


FIGURE 5.15. Toxicity effects of C18/Silica/Isolute PAH HC extracts on *S. typhimurium* grown in nutrient broth in a 96 well microtitre plate (n = 3). %CV ranges from 1.5-24.1%.

Results for the Blackburn extracts of oil 8, Nytro-10GBN, 4 and white oil in Figure 5.14 showed no decrease in absorbance of >20% at 50 mg well^{-1}. For 75 mg well^{-1} however, the absorbance shows a decrease of ~20% for oils 4 and Nytro-10GBN suggesting toxicity may have altered the Ames test results if a higher dose had been tested. Figure 5.15 indicates no conclusive reduction in absorbance for the C18/Silica/Isolute PAH HC extracts of white oil, oil 4 and Nytro-10GBN. For oil 8, toxicity could not be concluded due to a greater standard deviation. As no toxicity was found at the doses tested with the Ames test, the reversion frequency was not measured (Section 1.3.2). Toxicity is further discussed in Section 7.5.2.

5.8.2 Toxicity of Aged Oil Extracts

The same toxicity tests were performed on aged oil extracts. At 50 mg well^{-1} no decrease in absorbance at >20% was observed for the Grimmer extracts with the exception of ALT 3 (Figure 5.16). However, this reduction in absorbance was not consistent with increasing dose, suggesting that it was due to experimental imprecision. The final dose of 75 mg well^{-1} gave an absorbance decrease of ~20%, suggesting that ALT 3 was possibly toxic at higher dose. Repeats could not be performed due to a lack of sample.

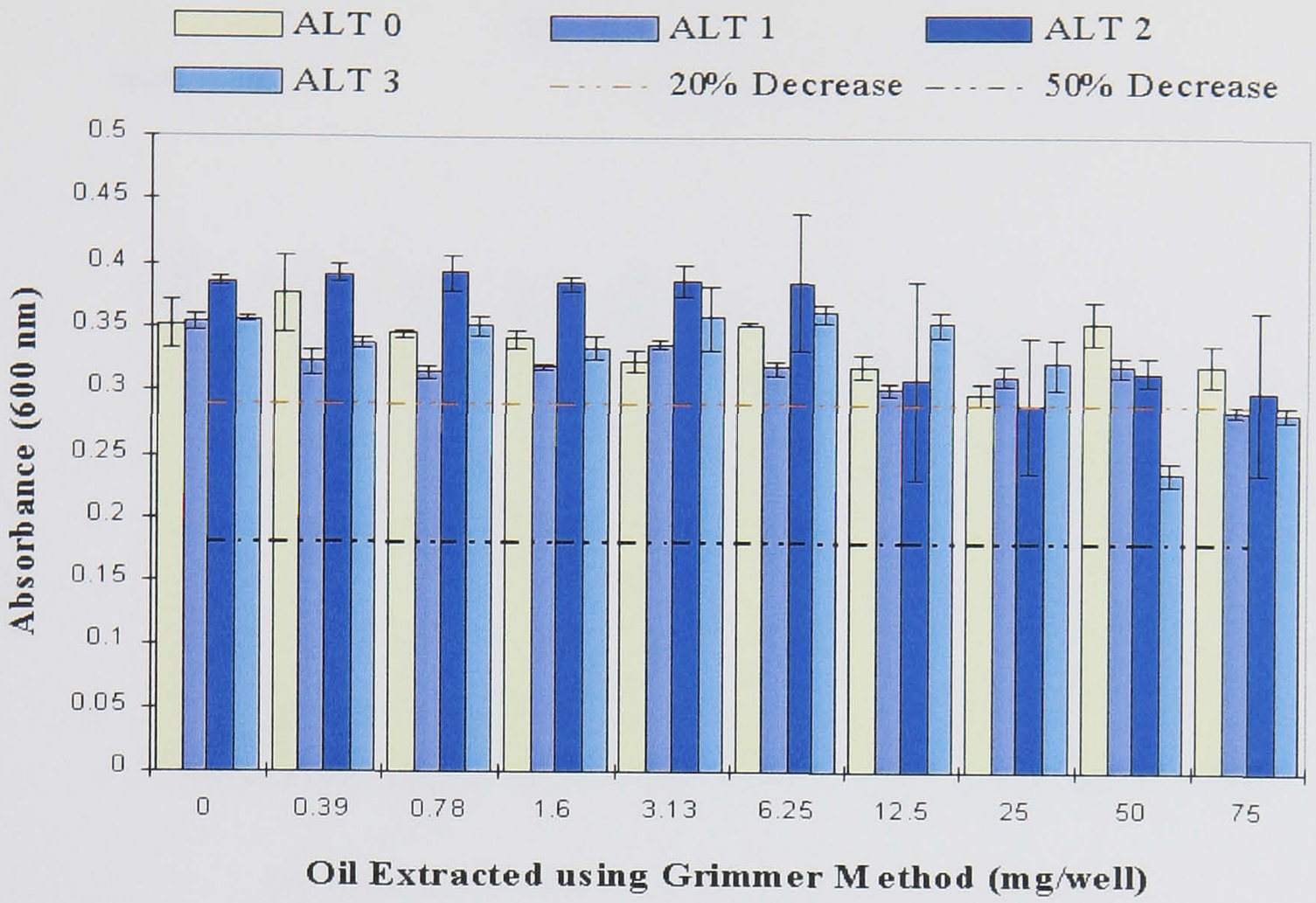


FIGURE 5.16. Toxicity effects of Grimmer extracts of aged oils on *S. typhimurium* grown in nutrient broth in a 96 well microtitre plate (n = 3). %CV ranges from 0.1-25.0%.

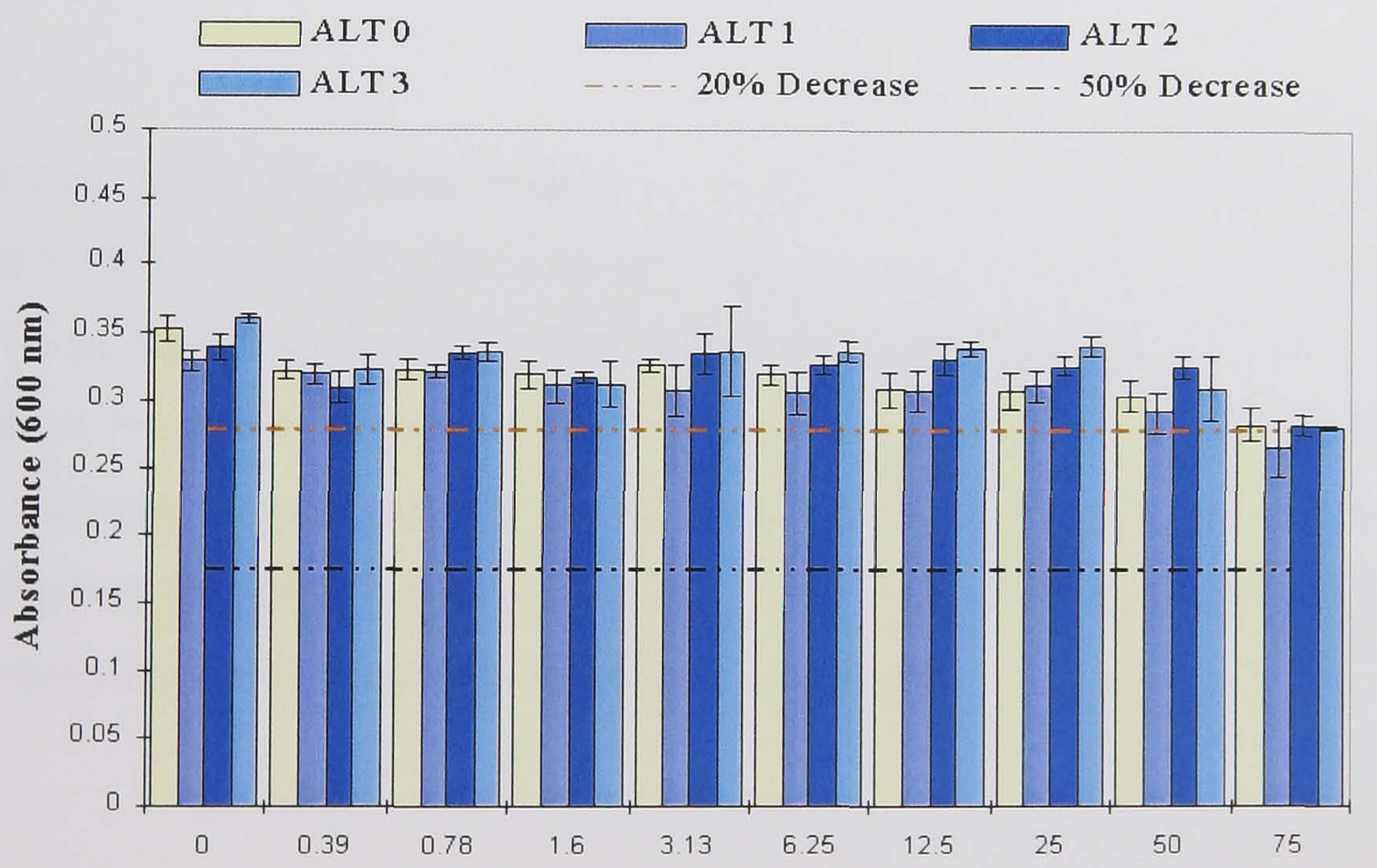


FIGURE 5.17. Toxicity effects of Blackburn extracts of aged oils on *S. typhimurium* grown in nutrient broth in a 96 well microtitre plate (n = 3). %CV ranges from 0.7-9.7%.

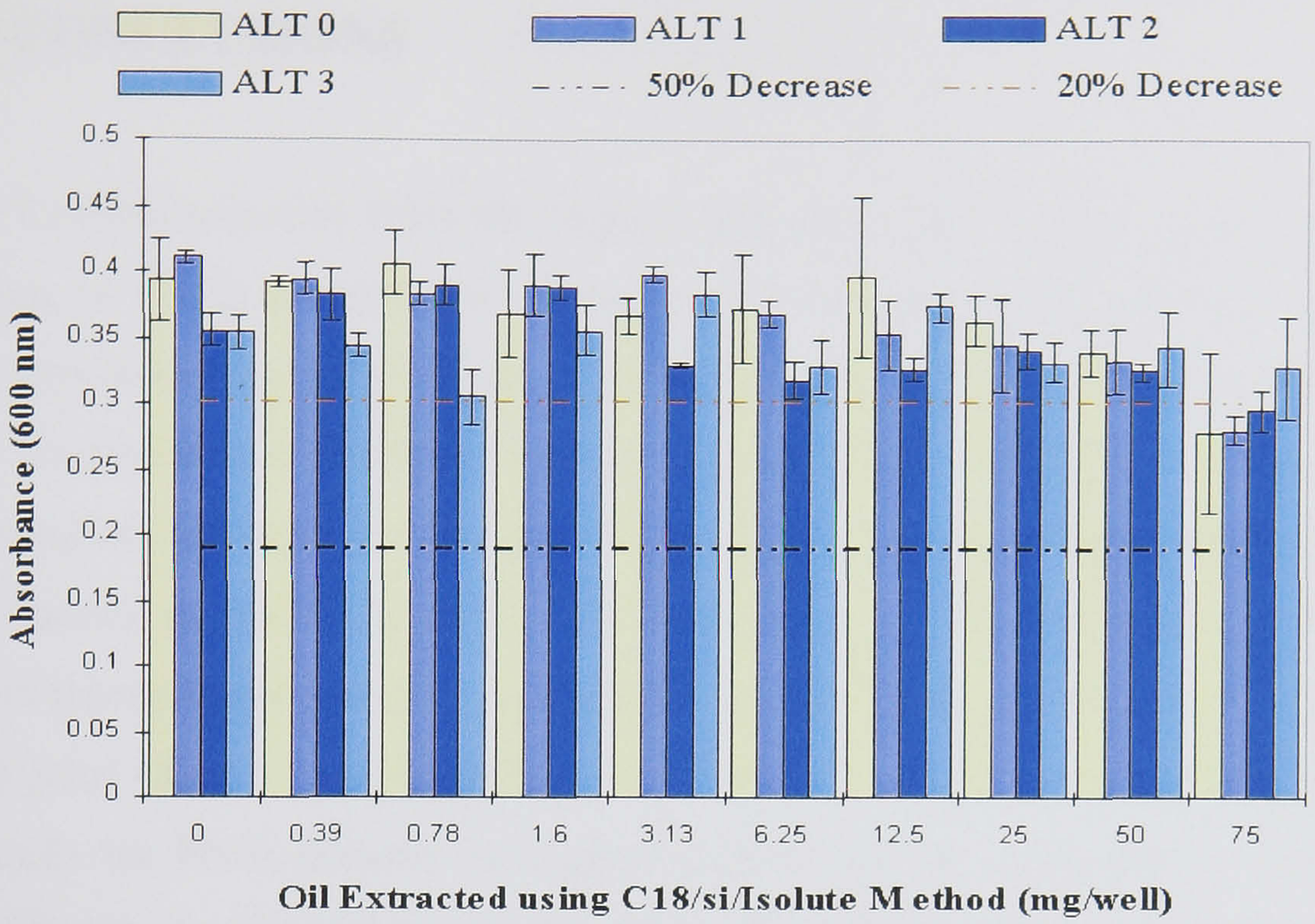


FIGURE 5.18 Toxicity effects of C18/Silica/Isolute PAH HC extracts of aged oils on *S. typhimurium* grown in nutrient broth in a 96 well microtitre plate (n = 3). %CV ranges from 0.6-21.7%.

In the case of the Blackburn (Figure 5.17) and C18/Silica/Isolute PAH HC extracts (figure 5.18) no oil produced a consistent reduction in absorbance of >20% at 50 mg well^{-1} so no significant toxicity effects could be established. However, at 75 mg well^{-1}, Blackburn extracts of all oils and C18/Silica/Isolute PAH HC extracts of oils ALT 1 and 2 indicated a possible toxic effect. Nevertheless, no clear, consistent toxic effect was observed for any oil at the 50 mg well^{-1} level, so potential toxicity effects on the Ames test were not conclusively observed for aged oils.

5.9 CONCLUSIONS

The C18/Silica/Isolute PAH HC extracts have been successfully used to determine the impact of PAHs on the total mutagenicity observed for transformer oils and with comparisons to the liquid-liquid extracts that measured total aromatic mutagenicity, it was concluded that PAHs are not the only mutagenic threat in oil. This justifies the measure of total aromatic content with the IP 346 % w/w method as an estimate of the oil's hazard to health. As PAHs still contribute to oil mutagenicity, and as they are an environmental pollutant, the development of the C18/Silica/Isolute PAH HC extraction is of great value. The C18/Silica/Isolute PAH HC extracts may be used to identify and quantify the PAHs present, as suggested by the results in Chapter 4. For this reason, the Chapter 6 will explore the use of the C18/Silica/Isolute PAH HC extracts as an alternative to immunoassay and IP 346 % w/w data.

CHAPTER 6.0

RESULTS

MEASURING VARIATION IN POLYCYCLIC AROMATIC HYDROCARBON CONTENT WITH THE C18 SILICA ISOLUTE EXTRACTION IN OILS

6.1 INTRODUCTION

6.1.1 Comparison of Qualitative Measurement

The primary function of developing a more PAH specific extraction method was to determine the mutagenicity risk of PAHs compared to total oil mutagenicity. In Chapters 3 and 5 it was found that PAH mutagenicity was not the only source of threat in the oils tested and that direct mutagen(s) were present in the aromatic fraction that were of equal, if not greater concern. This finding has established that measuring total aromatic content by the IP 346 % w/w method rather than PAH content is justified as all sources of mutagenicity seem to originate from the aromatic fraction of the oil.

As PAHs are environmental pollutants and do contribute to oil mutagenicity, the development of the C18/Silica/Isolute PAH HC extraction is still of great importance. The method was both repeatable and sufficiently efficient to allow quantification of the PAHs. Moreover, this method could be performed to identify unknown PAHs in the sample for an indication of possible mutagenicity. The National Grid Company Plc. (Pahlavanpour and Wilson, 1999) had previously identified the PAHs in transformer oils using the fingerprinting method developed by Wilson and Pahlavanpour (2000). Nytro-10GBN was one of the oils analysed with the fingerprinting technique so could be compared with the C18/Silica/Isolute PAH HC method to indicate the extraction capability of the new method. Unfortunately, the Wilson and Pahlavanpour (2000) method proved difficult to repeat due to the crude initial liquid-liquid extraction step, so quantitative data proved difficult to compile. For this reason, as well as being an easier, safer and more quantitative extraction method, the C18/Silica/Isolute PAH HC method offers significant analytical benefits.

6.1.2 Comparison of Total Aromatic Measurement

As the IP 346 % w/w extraction is a laborious method that measures aromatic content instead of polyaromatics (Stang, 1993; 1999) the C18/Silica/Isolute PAH HC extraction will be compared to the IP 346 % w/w method to determine if it could be used as an alternative or replacement method. Although the C18/Silica/Isolute PAH HC method should be more specific to PAHs, it was possible that the two measures were related. In order to compare the IP 346 % w/w data with C18/Silica/Isolute PAH HC data, the total peak area of the GC-MS chromatograms of the C18/Silica/Isolute PAH HC extracts were measured.

Two approaches were examined with regard to determining the total PAH content by the C18/Silica/Isolute PAH HC method. The first uses a measure of the total peak area of a total ion chromatogram (TIC) of a series of oils to indicate a trend in PAH content. For example, oil 8 which was estimated by IP346 to have a very high aromatic content (8.9% w/w), should have an area 8 times greater than oil 4 which was low in aromatics (<1% w/w). Oil Nytro-10GBN according to IP346 should have an area that falls in between these two extremes. The peak area at 21 minutes was not included as it was believed to be a contaminant from the extraction process. The second approach uses a selective ion chromatogram (SIC) instead of the TIC. Only the major molecular ions of the EPA 16 priority PAHs would be monitored with SIC but the methylated and unmethylated EPA 16 priority PAHs would also be included, as they all contain the molecular ions of the EPA 16 priority PAHs.

The C18/Silica/Isolute PAH HC extraction method and IP 346 % w/w values were also compared to a commercially available total PAH immunoassay kit. The immunoassay kit is specific to PAHs rather than total aromatic content, and has been used to measure PAH content in oils (Kim *et al.,* 2001). The kit is of interest to the National Grid Company Plc but is currently too expensive to be used in routine PAH detection. It was postulated that the C18/Silica/Isolute PAH HC extraction would correlate with the

PAH specific immunoassay data rather than the IP 346 % w/w data, and therefore be used as an alternative to immunoassay detection.

Additionally, a second immunoassay kit was also used to measure carcinogenic PAHs. This would relate carcinogenic PAH content to total PAH content. This would determine if total PAH detection could indicate carcinogenic risk without the use of mutagenicity testing (for screening purposes).

6.2 IMMUNOASSAYS AS A QUANITATIVE DETECTION METHOD

As IP 346 % w/w data was already available, no further extractions were required to correlate total aromatic content with the C18/Silica/Isolute PAH HC extraction method. However, to correlate total PAH content, immunoassay data had to be generated. Two commercial kits for the analysis of PAHs in water and soil were examined. The first measured total PAH content, that may be directly compared to IP346 measurements. The second had greater affinity for carcinogenic PAHs present in the sample. This carcinogenic kit was also evaluated as it was able to detect carcinogenic PAH levels below that of the Ames test. By measuring the carcinogenic PAH content, links may be established to the total PAH content. This would determine if a measure of total PAH content, such as the IP 346 % w/w method was an effective indication of carcinogenicity.

These kits have been used for the detection of PAHs in oil (Kim *et al.,* 2001) with results indicating that immunoassays may be more sensitive and selective for PAH detection, than the IP346 % w/w method.

6.2.1 Effect of Environment on Immunoassay

Both total and carcinogenic immunoassay kits were used to determine PAH content. The recommendation of the manufacturers (SDI) was that samples were made up in water, with a maximum of 25% v/v methanol. Organic solvent was used for dilution, with the final dilution in diluent.

Diluent (a buffered saline) from the kit was limited, so alternative buffer solutions were investigated as a blank. It can be seen in Table 6.1 that the recommended environment of water with 25% v/v methanol gave a reading of 5.8 $\mu g\ L^{-1}$. This error was small, but the sample dilution would have multiplied this imprecision. In addition, all other buffer solutions introduced further error. Table 6.1 shows that only using 100% diluent (from the kit) gave an error free reading. For this reason the percentage of diluent in the final dilution must be maximised. The level and type of organic solvent most compatible with the assay was also tested since the dilution of standard PAH samples with diluent gave imprecise results. This was believed to be due to PAH preference for an organic (as opposed to an aqueous) environment. After dilution of samples in various solvents, a washing of the dilution vessel yielded the following data: 100% diluent ~18% phenanthrene remaining in the vessel; 1% v/v methanol ~7%; 10% v/v methanol ~4%. All samples were therefore measured in a final dilution solution of 10% v/v methanol.

TABLE 6.1. The affect of solvent conditions on the blank response of the PAH immunoassay.

Sample	*PAH Reading from graph ($\mu g\ L^{-1}$)*
Water with 25% v/v methanol	5.8
100% phosphate buffer ph7.4	4.8
Phosphate buffer with 10% v/v acetone	34
Phosphate buffer with 1% v/v acetone	8.0
Phosphate buffer with 10% v/v methanol	12.0
Phosphate buffer with 1% v/v methanol	5.0
100% buffered saline ph7.4	2.4
Buffered saline pH 7.4 with 1% v/v acetone	5.2
Buffered saline pH 7.4 with 10% v/v acetone	8.6
Buffered saline pH 7.4 with 1% v/v methanol	2.4
Buffered saline pH 7.4 with 10% v/v methanol	8.0
50% buffered saline pH 7.4 with 50% v/v diluent	2.2
10% buffered saline pH 7.4 with 90% v/v diluent	1.1
100% diluent from kit	0
Diluent with 1% v/v acetone	2.4
Diluent with 10% v/v acetone	4.0
Diluent with 1% v/v methanol	1.2
Diluent with 10% v/v methanol	2.6

6.2.2 Testing the Immunoassay Kits for Accuracy

Both the total PAH kit and the carcinogenic PAH kit were tested for accuracy by using a solution of phenanthrene (total PAH kit) and benzo[a]pyrene (carcinogenic PAH kit). The solutions were made in-house to determine the imprecision likely when diluting real samples rather than the control solutions provided in the kit. A calibration curve was constructed for each kit. Measured values were compared to the expected result to determine if the kit was accurate at the range of detection stated by the manufacturer.

6.2.2.1 Total Immunoassay Kit

Figure 6.1 shows the calibration curve for the total PAH kit. Predictably, the largest deviation occurred at the detection limits of the system. This could not be due to systematic error from the dilution as no systematic increase in error was observed with dilution. The kit was most reliable at the 25 μg L^{-1} level, the mid-point of the assay range. This indicated that the most accurate results would be obtained by diluting the samples to an approximate concentration at the mid-point of the assay range.

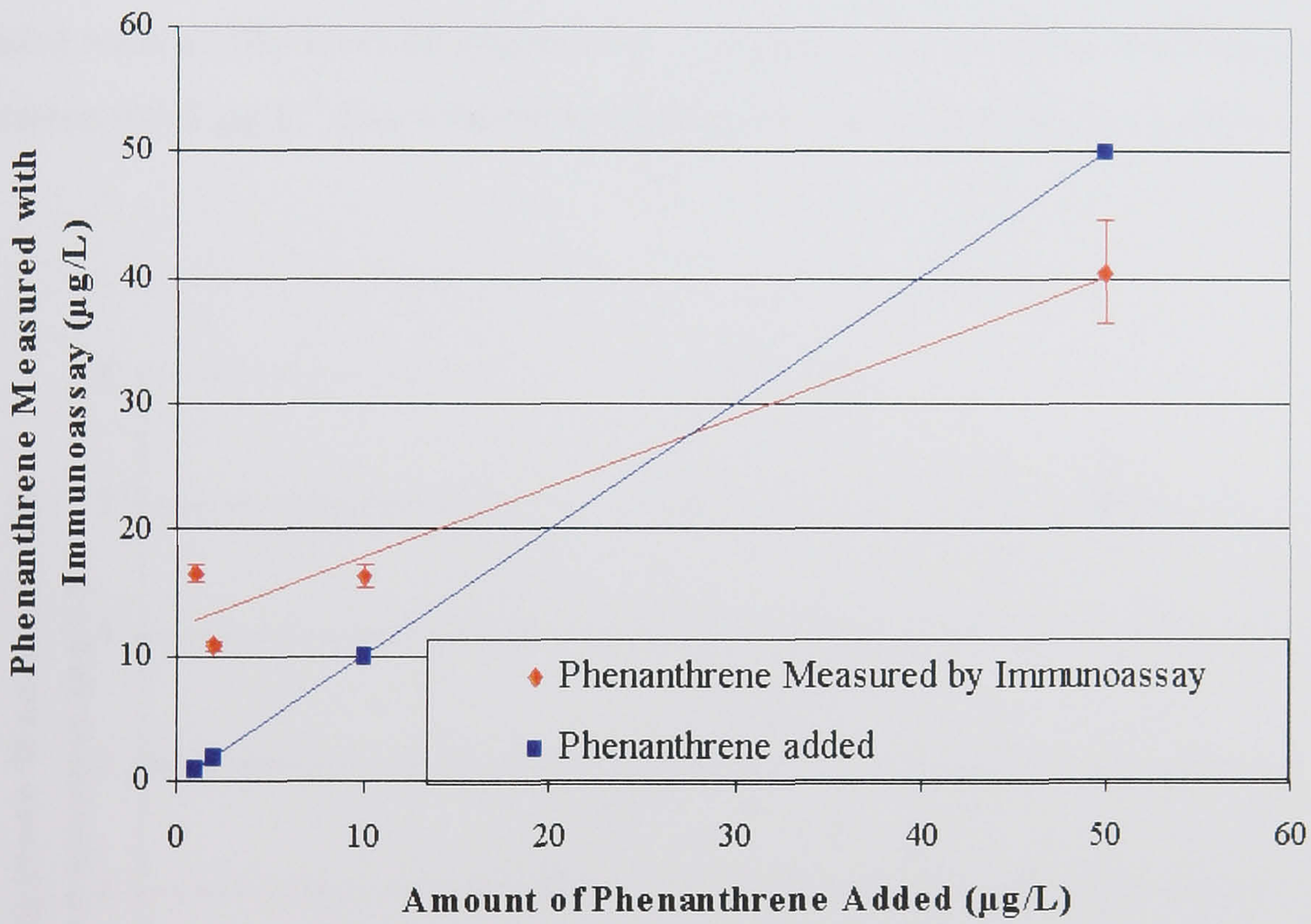


FIGURE 6.1. Phenanthrene solution prepared in-house to determine the accuracy of the total PAH kit within the range stated by the manufacturer (n = 3).

This made measurement difficult for samples with unknown PAH concentration. Many repeats over a range of dilutions is expensive to undertake, at a cost of ~£10 per sample and with the additional cost of £100 whenever the standards and controls were performed. This made the immunoassay kits impractical for the end-user. For this

reason, the test range was extended to between 10-40 μg L^{-1}.and a 10% level of imprecision was deemed acceptable.

6.2.2.2 Carcinogenic Immunoassay Kit

Figure 6.2 shows the calibration curve for the carcinogenic PAH kit. The largest deviation occurs at the lower limits of the assay. This was concluded to be due to systematic factors as the error increased with dilution. Systematic error may be improved with better dilution technique but error could be further minimised if measuring within the range of between 3-5 μg L^{-1}. Again, the accepted test range was extended with a 10% level of imprecision, yielding a carcinogenic kit detection range to between 0.5-5 μg L^{-1} that was more practical for use with unknown samples.

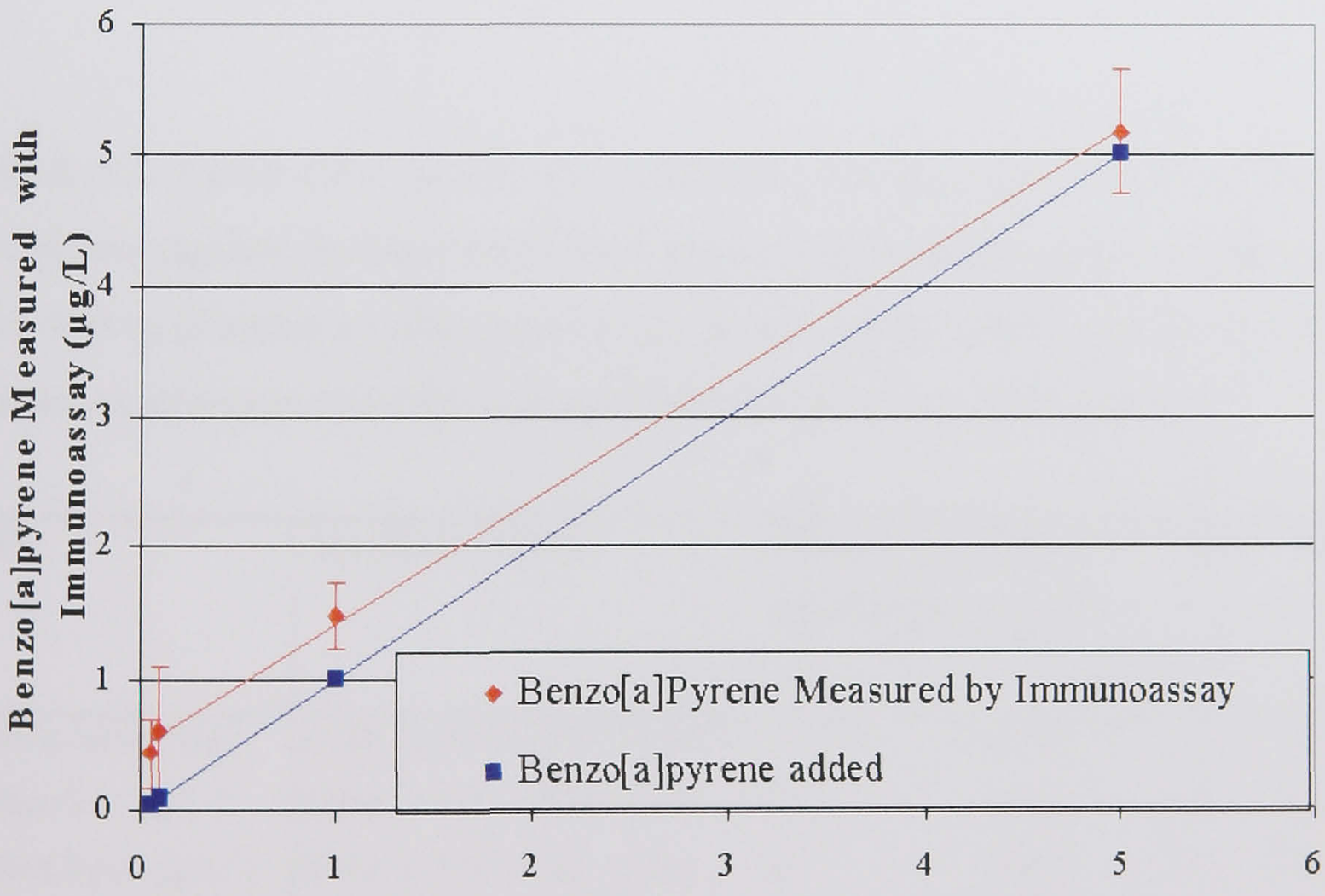


FIGURE 6.2. Benzo[a]pyrene solution prepared in-house to determine the accuracy of the carcinogenic PAH kit within the range stated by the manufacturer (n = 3).

6.2.3 Total and Carcinogenic PAH Content of Oils with Immunoassay

The total and carcinogenic PAH content of oils 1 to 12 were measured with the immunoassay kits as described in Section 2.2.13. The oils were diluted to a range that would allow measurement within the most accurate range of the kits (determined in Section 6.2.2). The data produced by the kit standards is recorded in Table 6.2 for the total immunoassay kit and Table 6.3 for the carcinogenic kit.

The $\%B/B_0$ of the standards were plotted against the amount of PAH ($\mu g\ L^{-1}$) on logarithmic graph paper supplied in the kit. The resulting linear calibration plot was used to determine the amount of PAH ($\mu g\ L^{-1}$) of the samples from their $\%B/B_0$ values. Data, including dilution factor, mean absorbance and B/B_0 values, are given in Table 6.4 for total PAH and Table 6.5 for carcinogenic PAH. Both oil 3 and oil 8 for the total PAH measurement, required further dilution (Table 6.4) as the PAH content of both was too high to be measured within the same range of the other oils.

TABLE 6.2. Calibration data for the total PAH immunoassay kit. The $\%B/B_0$ values were plotted against the amount of PAH ($\mu g\ L^{-1}$) on logarithmic graph paper (included in the kit) to produce a calibration curve on which the $\%B/B_0$ values of the samples could be used to determine the amount of PAH ($\mu g\ L^{-1}$) in each sample.

Sample	*Absorbance 450nm*				*Mean Absorbance (B)*	*± SD (n = 4)*	*%CV*	*%B/B₀*
Negative control (B_0)	1.186	1.189	1.158	1.154	1.1*	0.018	1.5	N/A
Standard 1 (2 $\mu g\ L^{-1}$)	0.956	0.959	1.052	1.038	1.0	0.050	5.0	85
Standard 2 (10 $\mu g\ L^{-1}$)	0.758	0.774	0.781	0.780	0.7	0.010	1.3	66
Standard 3 (50 $\mu g\ L^{-1}$)	0.523	0.513	0.499	0.486	0.5	0.016	3.2	43
Control (25 $\mu g\ L^{-1}$)	0.607	0.599	--	--	0.6	0.005	0.9	51

* *B_0 value*

TABLE 6.3. Calibration data for the carcinogenic PAH immunoassay kit. The $\%B/B_0$ values were plotted against the amount of PAH ($\mu g\ L^{-1}$) on logarithmic graph paper (included in the kit) to produce a calibration curve on which the $\%B/B_0$ values of the samples could be used to determine the amount of PAH ($\mu g\ L^{-1}$) in each sample.

Sample	*Absorbance 450nm*				*Mean Absorbance (B)*	*± SD (n = 4)*	*%CV*	*%B/B₀*
negative control (B_0)	0.715	0.714	0.714	0.719	0.71*	0.002	0.3	N/A
standard 1 (0.1 $\mu g\ L^{-1}$)	0.650	0.652	0.645	0.654	0.65	0.003	0.5	91
standard 2 (1 $\mu g\ L^{-1}$)	0.433	0.444	0.447	0.439	0.44	0.006	1.3	62
standard 3 (5 $\mu g\ L^{-1}$)	0.278	0.281	0.268	0.286	0.27	0.007	2.7	39
Control (2 $\mu g\ L^{-1}$)	0.388	0.382	--	--	0.38	0.003	0.8	54

** B_0 value*

TABLE 6.4. Data for the total PAH immunoassay kit for transformer oils 1 to 12.

Transformer Oil	*Dilution Factor*	*Mean Absorbance (B)*	*± SD (n = 4)*	*%CV*	*%B/B0*
1	10^{-5}	0.63	0.007	1.1	54
2	10^{-5}	0.49	0.007	1.4	42
3	10^{-6}	0.91	0.004	0.4	78*
4	10^{-5}	0.90	0.004	0.4	77
5	10^{-5}	0.49	0.005	1.1	42
6	10^{-5}	0.62	0.014	2.2	53
7	10^{-5}	0.68	0.009	1.3	58
8	10^{-6}	0.67	0.016	2.3	58*
9	10^{-5}	0.57	0.015	2.7	49
10	10^{-5}	0.48	0.010	2.2	41
11	10^{-5}	0.67	0.016	2.3	57
12	10^{-5}	0.51	0.005	0.	66

** Due to high PAH content, these oils were diluted further so that an absorbance reading could be obtained that fell within the range of the immunoassay kit. For this reason, although the $\%B/B_0$ values look similar to the other oils, their content is 10 times that of oil with a similar $\%B/B_0$ value.*

TABLE 6.5. Data for the carcinogenic PAH immunoassay kit for transformer oils 1 to 12.

Transformer Oil	*Dilution Factor*	*Mean Absorbance (B)*	*± SD (n = 4)*	*%CV*	*%B/B0*
1	10^{-6}	0.63	0.010	1.5	89
2	10^{-6}	0.54	0.012	2.3	77
3	10^{-6}	0.63	0.005	0.9	88
4	10^{-6}	0.68	0.008	1.2	96
5	10^{-6}	0.58	0.009	1.6	81
6	10^{-6}	0.59	0.006	1.0	83
7	10^{-6}	0.64	0.005	0.8	90
8	10^{-6}	0.50	0.006	1.2	71
9	10^{-6}	0.63	0.005	0.8	88
10	10^{-6}	0.57	0.007	1.2	81
11	10^{-6}	0.67	0.009	1.3	94
12	10^{-6}	0.61	0.005	0.9	86

Figure 6.3 shows a comparison of the total and carcinogenic PAH content of the twelve transformer oils. The carcinogenic data was multiplied by a factor of 33, (mean difference between the two sets of data) to allow comparison of the two test kits. This allowed a better determination of the trends observed for the twelve oils.

The general trend observed was that carcinogenic content of an oil increased with overall PAH content. The exception was oil 4 which contained a much lower carcinogenic PAH content than would be estimated by the trend, possibly due to lying outside the dynamic range of the assay. As 11 of the 12 oils followed the trend, it was considered a significant finding in that the measure of total oil PAH content by the IP 346 % w/w method, immunoassay or total GCMS spectral integration were potentially valid methods of assessing carcinogenic threat.

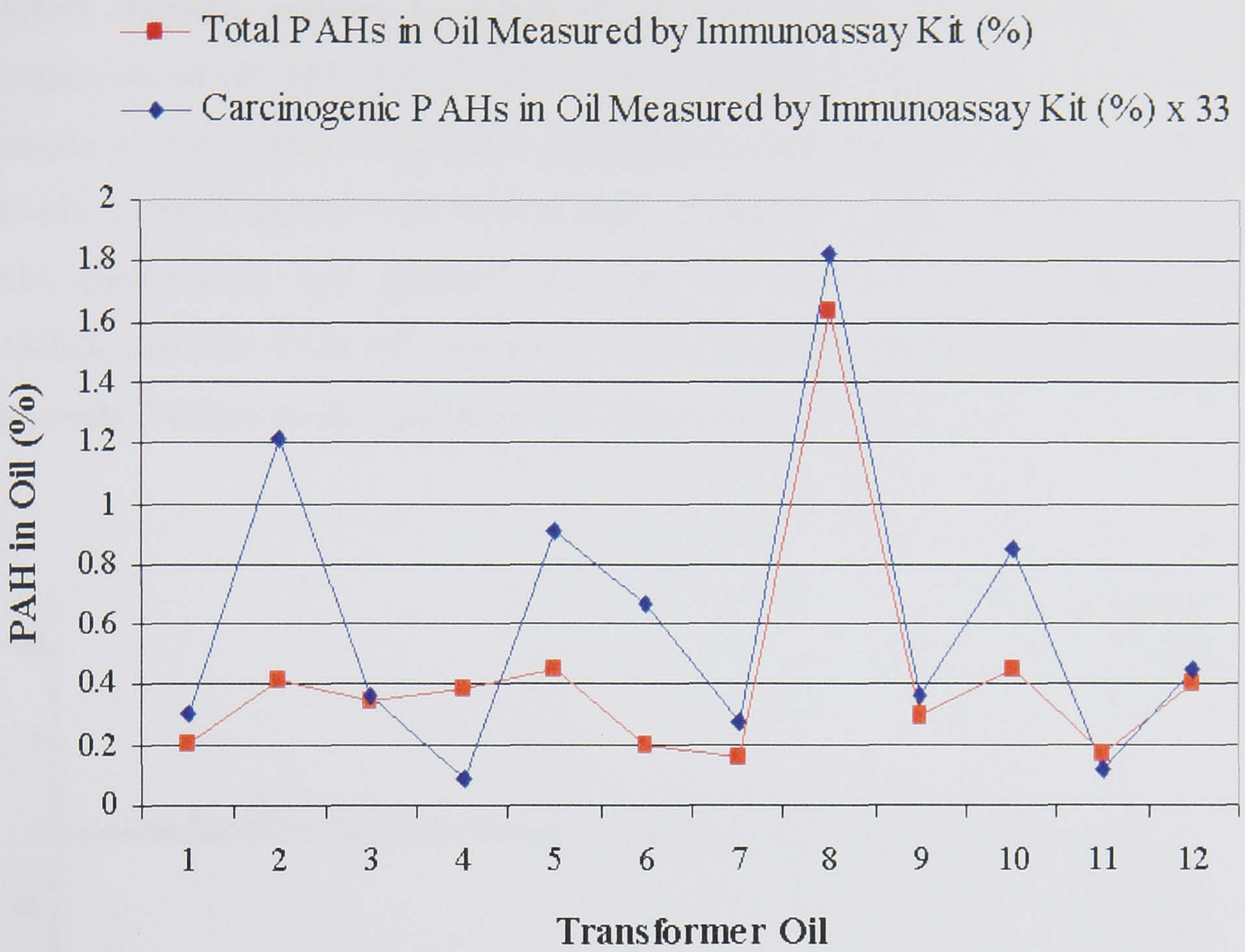


FIGURE 6.3. Comparison of the percentage total and carcinogenic PAHs detected in transformer oils 1 to 12. Carcinogenic PAH data has been multiplied by a factor of 33 to allow comparison of the data on the same scale. Error bars are not shown as the repeated samples shown in Table 6.2 and 6.4 were combined to give mean readings of PAH content.

6.3 GENERAL TRENDS IN PAH CONTENT OF TRANSFORMER OILS

Oils 1 to 12 (100 µL) were extracted with the small scale C18/Silica/Isolute PAH HC method (Section 2.2.9). The TIC chromatograms obtained varied according to their PAH composition and were used to identify and quantify PAHs. From a simple examination of the chromatograms, it was observed that the oils yielding a higher aromatic content by IP 346 % w/w, yielded more complex chromatograms, as can be seen for oils 10, 4 and 8 in Figure 6.4. As shown in Table 2.1, oil 8 contained the

greatest aromatic content according to IP 346 % w/w data and proved the most complex oil on GC-MS analysis (Figure 6.4 and 6.5). In turn, oil 10 contained a higher quantity of PAH species than oil 4 indicating a correlation in trend for C18/Si/Isolute GC-MS extracts and IP 346 % w/w data. However, a more detailed comparison of PAH composition and quantity with IP 346 % w/w data, immunoassay and C18/Silica/Isolute PAH HC extraction was established before a definite conclusion was made. Further work is detailed in Section 6.3.1.

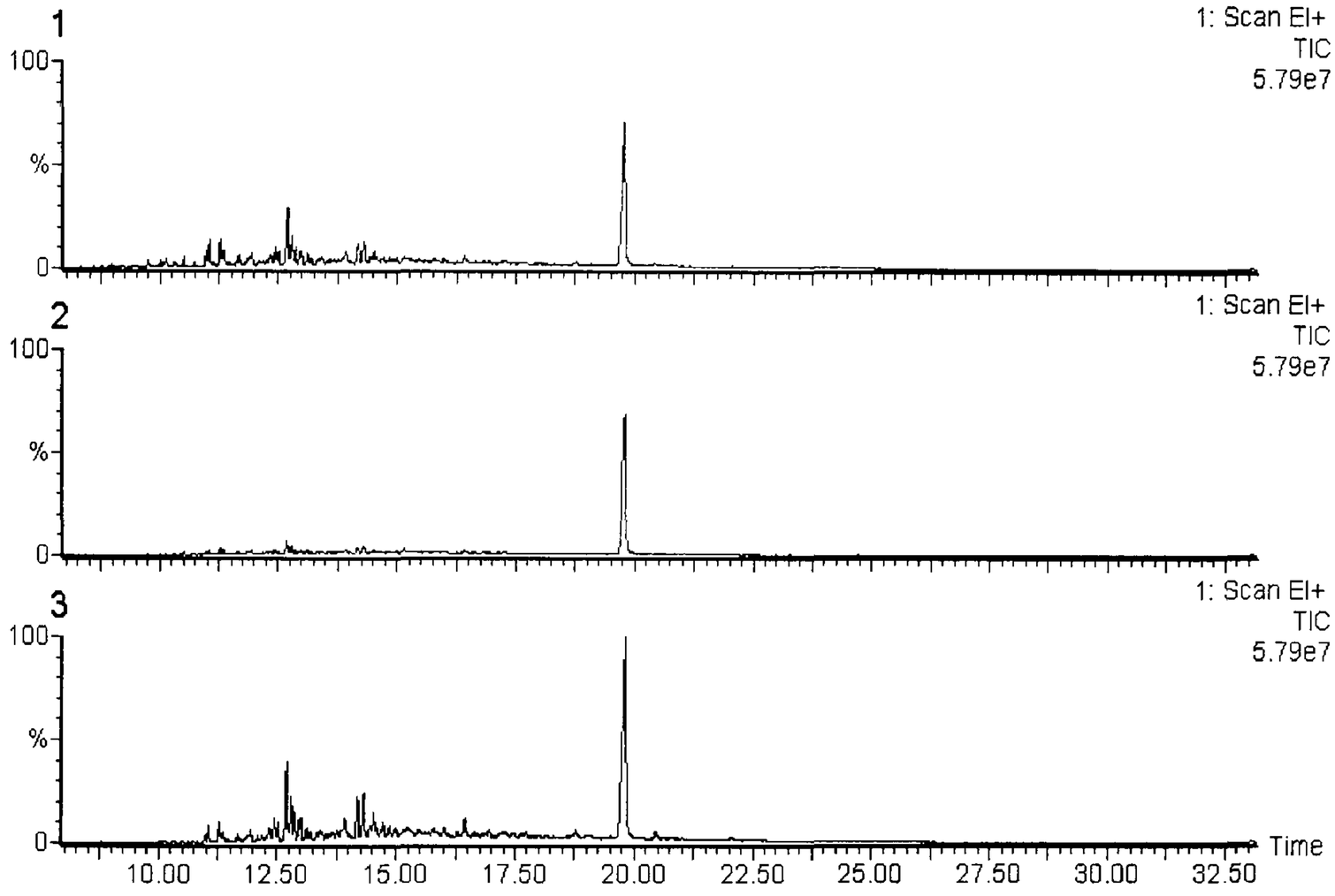


FIGURE 6.4. The TIC of oils extracted by the C18 Silica Isolute PAH HC method. 1 = oil 10 (IP 346 % = 4.5), 2 = oil 4 (IP 346 % = <1), 3 = oil 8 (IP 346 % = 8.9). Spectra run on the Perkin Elmer Turbomass (Section 2.2.6).

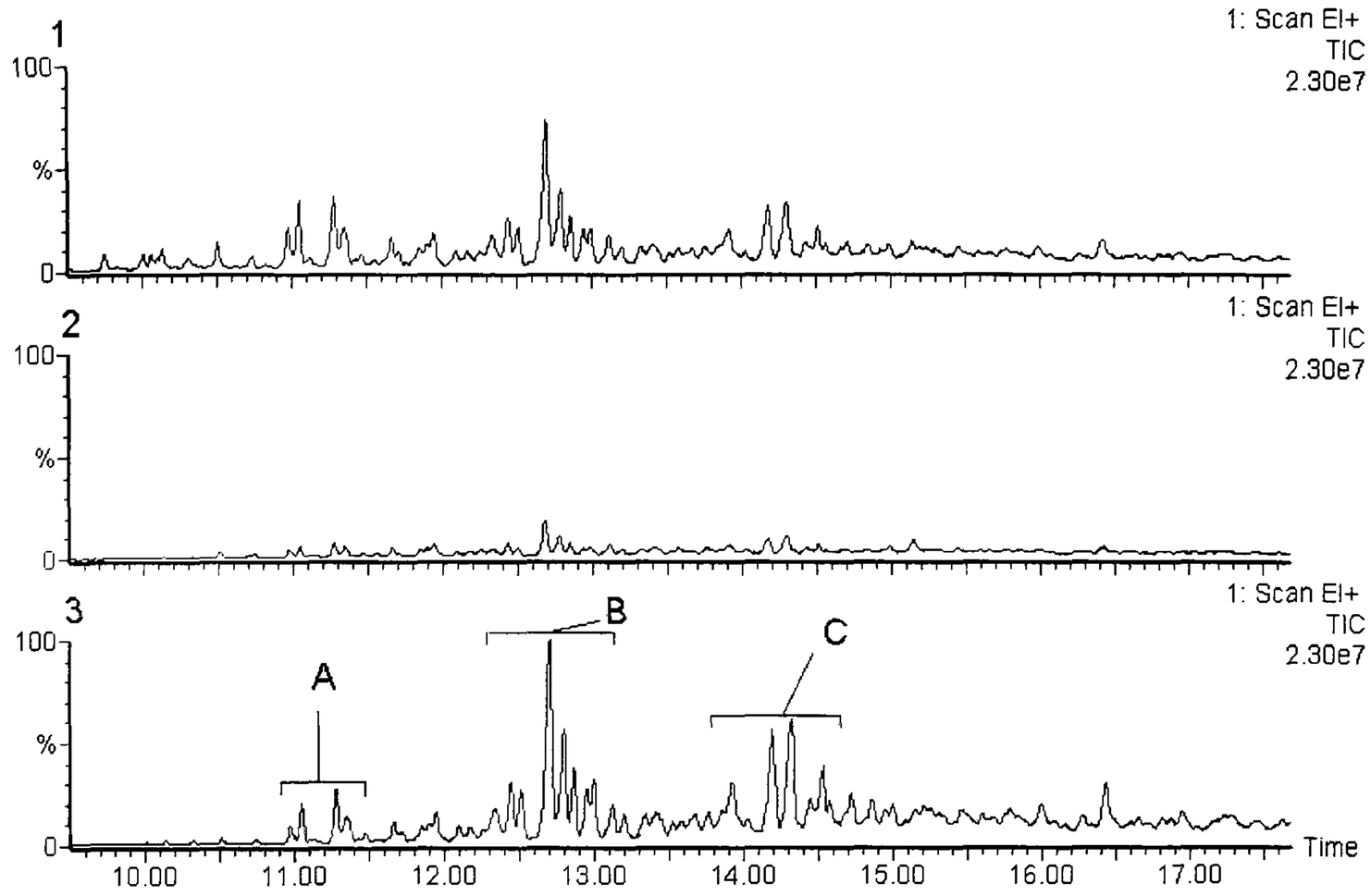


FIGURE 6.5. A close up of the first 17 minutes of the spectra shown in Figure 6.4 extracted by the C18 Silica Isolute PAH HC method. 1 = oil 10, 2 = oil 4, 3 = oil 8. A = Phenanthrene, B = Phenanthrene methyl isomers, C = Phenanthrene dimethyl isomers.

6.3.1 Validation of the C18 Silica Isolute PAH HC Extraction Method for Total PAH Determination

Due to the laborious and crude nature of the IP 346 % w/w method it was advantageous to find an alternative method of measuring polyaromatic content. The immunoassay method offered an obvious alternative, as it measured total PAH in a sample. This method had its disadvantages however, as the immunoassay kits were considered too expensive in the long term for the end-user.

As the C18/Silica/Isolute PAH HC method extracts a range of representative PAHs present in the oil, it may be possible to use this method as an alternative to IP 346 or immunoassays. As seen in Section 6.3, the chromatograms of oils with a high PAH

content tend to be more complex, and the summing of the peak areas of a whole chromatogram should be much greater than those oils of low PAH content. A comparison of total peak area was therefore used to compare the oils according to PAH content. This is shown in Figure 6.6 along with the total PAH immunoassay data and the IP 346 % w/w data. Total integration of the selective ion chromatogram was also compared. The raw data from both TIC and SIC integration is compiled in Appendix D. The immunoassay and IP 346 % w/w data is given as milligram of PAH per mL of oil (mg mL^{-1}). The immunoassay data was multiplied by a factor of 7.1 (the mean difference between immunoassay data and IP 346 % w/w data) purely to allow direct method comparison on the same scale.

The TIC data was compared to the immunoassay, SIC and IP 346 % w/w data in turn to determine if the C18/Silica/Isolute PAH HC TIC peak integration could be used as an alternative to each method:

1. **TIC vs. Immunoassay.** The data for TIC integration and immunoassay in Figure 6.6 followed a similar trend for most oils, although the extent to which each oil varied was more exaggerated using the immunoassay method. In addition, the PAH content for oil 4 (3.14×10^6 peak area) was less than oil 3 (3.86×10^6 peak area) with TIC integration, but the immunoassay methods yielded a higher PAH content for oil 4 (4.2 mg mL^{-1}) than oil 3 (3.8 mg mL^{-1}).

2. **TIC vs. SIC.** SIC correlated with TIC data with the exception of oil 2 and 3. SIC data showed oil 2 and 3 to have a lower PAH content than oil 1. However, both the TIC and immunoassay data showed oil 1 to have the lowest. This suggested that the integration of a TIC was more accurate than the integration of a SIC for determining the trend in oil PAH content.

3. **TIC and IP 346.** IP 346 data followed a similar trend to TIC data, although the TIC trend was not as exaggerated as the IP 346 trend. For example, the TIC data for oil 8 was not as different from all other oils as IP 346 or immunoassay data.

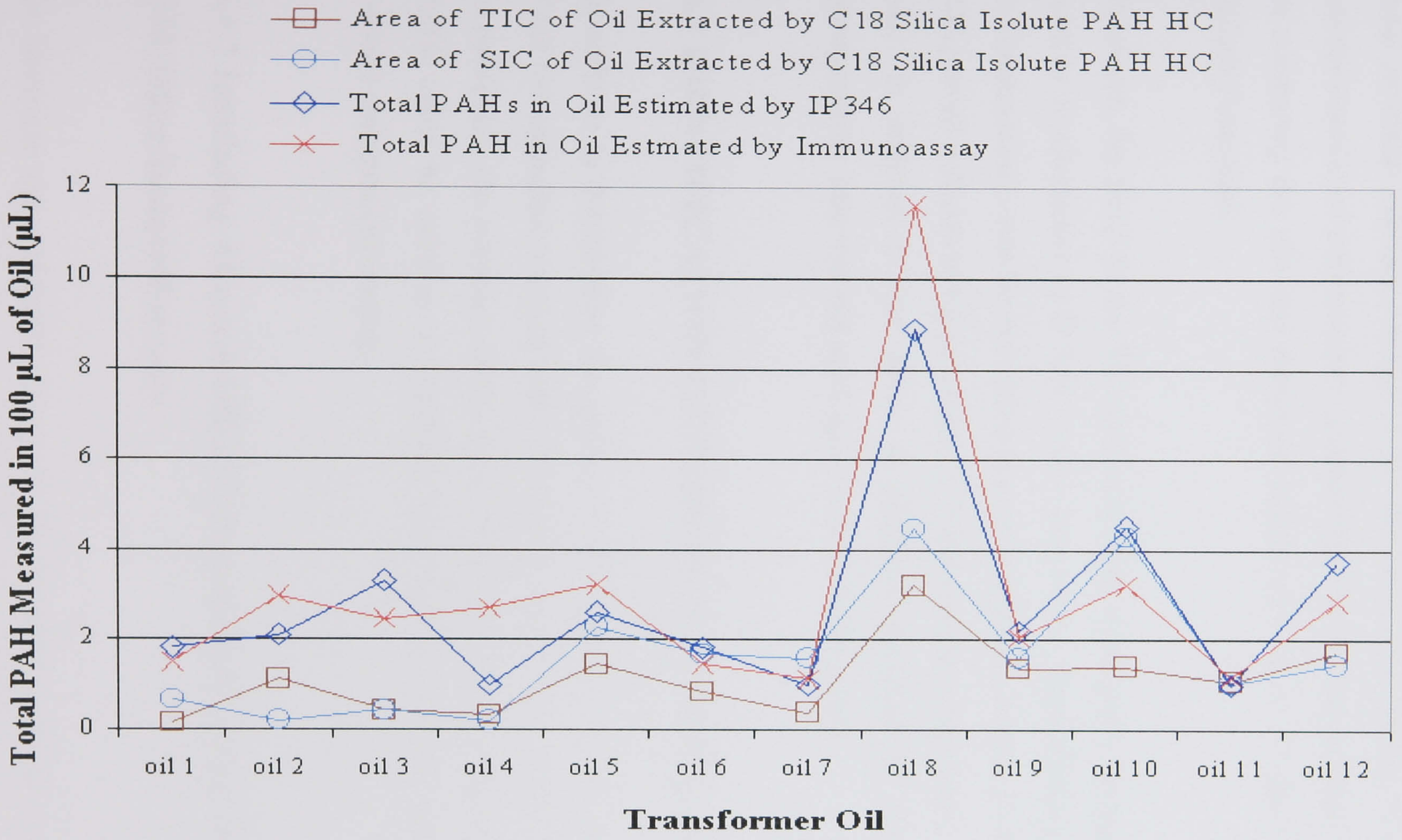


FIGURE 6.6. IP 346, total immunoassay, GCMS TIC integration and GCMS SIC integration compared for transformer oil 1 to 12. Data has been manipulated to scale all data for comparison. Areas of TIC and SIC are 10^9; IP 346 data is a percentage value (w/w); Immunoassay is given as a percentage (w/v) and multiplied by a factor of 7.1.

The IP 346 % w/w method, although used to determine the polyaromatic content of an oil, is known to extract total aromatic content (Stang, 1993; 1999). As the C18/Silica/Isolute PAH HC TIC integration correlated with both IP 346 and immunoassay, it was clear that total PAH content (measured by immunoassay) and total aromatic content (extracted by IP 346) were linked. This suggests that the overestimation of polyaromatic content with the IP 346 method is of no consequence in comparing the oils and therefore justifies the choice of the IP 346 method as the industry standard.

However, the trend in the TIC data correlates sufficiently to suggest that it could be used as an alternative to IP 346 % w/w data for the estimation of polyaromatic content or immunoassay data for the estimation of total PAH content. This was beneficial as IP 346 proved difficult to repeat (Table 3.7) and immunoassay was expensive for the end-user. In addition it was easier to perform, therefore IP 346 % w/w data and immunoassay data was not used again in this investigation.

6.4 IDENTIFICATION OF PAHS IN TRANSFORMER OIL

In addition to determining the general PAH content of an oil, the C18/Silica/Isolute PAH HC extraction method with GC-MS, can be used to identify and quantify PAHs. In particular, the presence of the EPA 16 priority PAHs and additional carcinogenic PAHs could be used as a screening technique to indicate potential health threats, without mutagenicity testing.

6.4.1 Insulating Mineral Oil Fingerprinting Compared to C18/Silica/Isolute Extracts

To determine the qualitative nature of the C18/Silica/Isolute PAH HC extracts, the PAHs identified with the fingerprinting technique were compared to those in the C18/Silica/Isolute PAH HC extract of oil Nytro-10GBN. Although the NIST library allowed the identification of PAHs in the samples, GC-MS could not determine which

isomers were present without the use of standards. As standards for each isomer proved to expensive to purchase for the identification of all the PAHs in the oil, identification did not go as far as the determination of isomers. For this reason Table 6.6 groups the isomers of each PAH found by the National Grid Company Plc and indicates which of these groups are present in the C18/Silica/Isolute PAH HC extract.

TABLE 6.6. Identification of PAHs in Nytro-10GBN C18/Silica/Isolute PAH HC extracts compared to those found by the National Grid Company Plc using the fingerprinting technique (Wilson and Pahlavanpour, 2000).

PAHs Identified by the National Grid Company Plc in Nytro-10GBN	*PAH Found in C18/Silica/Isolute PAH HC spectrum*
1, 1'-Biphenyl	Yes
1, 1'-Biphenyl, 2-methyl 1, 1'-Biphenyl, 3-methyl 2, 2'-Dimethyl-1,1'-biphenyl 3, 3'-Dimethyl-1,1'-biphenyl	Yes No
1, 1'-Biphenyl, 4, 4-dimethyl	No
Naphthalene, 1, 4, 6-trimethyl- Naphthalene, 1, 4, 5-trimethyl-	No
9-H-fluorene 3-methylfluorene 9-H-Fluorene, 2, 3-dimethyl	Yes Yes Yes
Phenanthrene	Yes
Phenanthrene, 9-methyl-	Yes
Phenanthrene, 4, 5-dimethyl- Phenanthrene, 2, 5-dimethyl- Phenanthrene, 2, 7-dimethyl-	Yes
Phenanthrene, 2, 3, 5-trimethyl-	Yes
Pyrene	Yes
Pyrene, 1-methyl Pyrene, 2-methyl	Yes
Pyrene, 1, 3-dimethyl	No

It can be seen that the C18/Silica/Isolute PAH HC extract did not contain the naphthalenes identified by the National Grid Company Plc. This absence was possibly due to loss during the evaporation steps of the C18/Silica/Isolute PAH HC extraction discussed in Section 4.6.1. Nevertheless, the most abundant PAHs, such as phenanthrenes and fluorene were present and suggested that the C18/Silica/Isolute PAH HC extraction method may be used for the identification of PAHs in oil.

6.4.2 Identification of PAHs in Oil 1 to 12

Oils 1 to 12 provided by the National Grid Company Plc were extracted using the C18/Silica/Isolute PAH HC method and the GC-MS TIC of each were analysed to determine PAH composition, which is recorded in Table 6.7.

It can be seen that a mixture of PAHs were present, although the majority were phenanthrene or fluorene based. However, no suggestion that these methylated PAHs were mutagenic was found in the literature as they are not well studied. It is assumed that they have the same mutagenicity as the parent PAH (Irwin, 1997).

Dibenzothiophene (including its methylated forms), benzo[g,h,i]fluoranthene, dimethyl benzo[c]phenanthrene, and methyl chrysene were of concern as they might impact on the observed mutagenicity in the oil. Dibenzothiophene, although strictly a heterocyclic compound and therefore not a PAH, was included due to its abundance in the extract and possible carcinogenic and toxic implications, although carcinogenic properties were not conclusive (Irwin, 1997).

No information on benzo[g,h,i]fluoranthene mutagenicity or carcinogenicity was found, but caution was applied due to inconclusive evidence in carcinogenic test (IARC, 2002). Benzo[c]phenanthrene was considered to be mutagenic with the Ames test (IARC, 2002) and therefore may be one of the sources of mutagenicity in oil 5, 6 and 8, although the carcinogenicity of dimethyl benzo[c]phenanthrene was not found in the literature.

TABLE 6.7. PAHs identified in oil 1 to 12 using the C18/Silica/Isolute PAH HC extraction method after analysis with GCMS TIC. The presence of a PAH is indicated by a coloured box.

PAHs Identified in Transformer Oil	*Retention Window (Minutes)*	*Oil 1*	*Oil 2*	*Oil 3*	*Oil 4*	*Oil 5*	*Oil 6*	*Oil 7*	*Oil 8*	*Oil 9*	*Oil 10*	*Oil 11*	*Oil 12*
fluorene, methyl-	8.8-9.0												
Fluorene, dimethyl-	10.0-10.5												
Phenanthrene	10.0-10.5												
Phenanthrene, methyl-	11.0-11.3												
Phenanthrene, dimethyl-	12.6-13.0												
Phenanthrene, trimethyl-	14.2-14.8												
Phenanthrene, tetramethyl-	16.2-16.5												
Pyrene	13.8-13.9												
Pyrene, methyl-	15.8-16.2												
Pyrene, dimethyl-	17.5-17.8												
Acenaphthylene, tetramethyl-	11.7-11.9												
Dibenzothiophene	9.4-9.6												
Dibenzothiophene, methyl-	10.5-11.0												
Dibenzothiophene, dimethyl-	11.5-12.0												
Benzo[g,h,i]fluoranthene	13.0-13.3												
Chrysene	18.7-18.8												
Chrysene, methyl-	20.4-20.6												
Benzo[c]phenanthrene, dimethyl-	22.0-22.4												

Methyl-chrysene was of concern as 5-methyl chrysene has been identified by the IARC as of carcinogenic risk (IARC, 2002) which is explained by the fact that this isomer is methylated in the bay region of the structure (Harvey, 1991) (Section 1.2.3). No other well known mutagen was found. This showed that either the mutagenic component of the oil had not been identified, and/or the mutagenicity was a product of the identified PAHs in combination, as the mutagenicity of such mixtures are not well understood in the literature.

6.5 QUANTITATION OF EPA 16 PRIORITY PAHS IN EACH OIL

Although a number of PAHs were identified, quantification of the EPA 16 priority PAHs was performed to identify levels of carcinogenic PAHs that may account for the indirect mutagenicity observed in the Ames test. SIC was analysed for the presence of the EPA 16 priority PAHs and quantified using the HP GC-MS with manual integration. Manual integration allowed very small peaks (that would usually be omitted by the software) to be identified. Samples were concentrated to 0.1 mL (as opposed to 1 mL) to improve the precision of the GC-MS analysis.

TABLE 6.8. Calibration data for the EPA 16 priority PAHs.

PAHs	R^2	$y = bx + a$	*Error Of Calibration Curve (%)*	*Limit of Detection ($\mu g\ mL^{-1}$)*
Naphthalene	0.994	73394x + 4371.7	11	0.21
Acenaphthylene	0.993	55658x + 3614.1	12	0.24
Acenaphthene	0.990	30666x + 4818.5	14	0.44
Fluorene	0.990	10341x + 4267.3	14	0.34
Phenanthrene	0.986	8512.5x + 3187.6	17	0.23
Anthracene	0.993	48911x + 5593.2	12	0.33
Fluoranthene	0.993	60267x + 3249.4	12	0.11
Pyrene	0.992	64196x + 3288.9	13	0.21
Benzo(a)anthracene	0.991	41021x + 856.35	13	0.16
Chrysene	0.987	26121x + 2928.2	16	0.35
Benzo(b)fluoranthene	0.993	28567x + 705.59	12	0.15
Benzo(k)fluoranthene	0.991	26876x + 1781.3	13	0.25
Benzo(a)pyrene	0.992	20161x + 1306.2	12	0.24
Indeno(1,2,3-c,d)pyrene	0.990	9767.8x + 490.55	14	0.22
Dibenz(a,h)anthracene	0.990	6740.6x + 1086.2	13	0.44
Benzo(g,h,i)perylene	0.991	10406x + 918.39	13	0.29

Table 6.8 shows the calibration data for each EPA 16 prority PAH. To improve precision the samples were tested in triplicate. Six extractions were performed simultaneously to increased the throughput of samples. As the HP GC-MS had to be manually injected, 1 $\mu g\ mL^{-1}$ deuterated chrysene was added to each sample after extraction but prior to GC-MS injection. The deuterated chrysene was quantified in the same way as the EPA 16 priority PAHs. The efficiency of the sample injection was therefore measured by quantifying the deuterated chrysene and a multiplication factor (Table 6.9) was used to correct for injection errors.

TABLE 6.9. Amount of internal standard recovered after injection with oil extracts of oils 1 to 12. Each extract was spiked with 1 µg ml^{-1} deuterated chrysene after extraction to determine the efficiency of injection into the GCMS. The multiplication factors were used to compensate for inefficient sample injection.

Oil	*Chrysene-D ($\mu g\ mL^{-1}$)*	*Multiplication factor*
1	0.56	1.7
2	0.83	1.2
3	0.71	1.3
4	0.86	1.1
5	0.43	2.3
6	0.26	3.8
7	0.34	2.9
8	0.78	1.2
9	0.10	9.8
10	0.56	1.8
11	0.56	1.8
12	0.56	1.8

The quantities of the EPA 16 priority PAHs identified in oils 1 to 12 are given in Table 6.10. Benzo[k]fluoranthene was identified in the SIC but was not present in quantities that could be quantified. This PAH is a known mutagen (IARC, 2002) and so could contribute to the indirect mutagenicity observed with the C18/Silica/Isolute PAH HC extracts in the Ames test.

Considering that the extraction efficiency of all PAHs was below 100%, the efficiencies previously calculated in Section 4.6 (Table 4.4) were used to determine the content of oil at 100% efficiency.

TABLE 6.10. The amount of the EPA 16 priority PAHs found in 100 μL of transformer oil. The results have been subject to the multiplication factors in Table 6.9 and multiplied to determine the amount of each PAH if the extraction had been 100% efficient (using extraction efficiencies in Table 4.4).

	PAHs in Transformer Oil (μg)											
PAHs	1	2	3	4	5	6	7	8	9	10	11	12
Naphthalene	0	0	0	0	0	0	0	0	0	0	0	0
Acenaphthylene	0	0	0	0	0	0	0	0	0	0	0	0
Acenaphthene	0	0	0	0	0	0	0	0	0	0	0	0
Fluorene	0	0	0	0	0.2	4.7	1.0	0	0	4.1	1.4	1.0
Phenanthrene	0.9	0	0.6	0	2.9	1.2	0.6	3.2	2.1	2.3	1.3	0.8
Anthracene	0	0	0	0	1.5	0	0	0.1	0.6	0	0.8	0
Fluoranthene	0	0	0	0	0	0	0	0	0	0	0	0
Pyrene	0	0	0	0	0	0	0	0	0	0	0	0
Benzo(a)anthracene	0	0	0	0	0	0	0	0.50	0	0	0	0
Chrysene	0	0	0	0	0	0	0	0	0	0	0	0
Benzo(b)fluoranthene	0	0	0	0	0	0	0	0	0	0	0	0
Benzo(k)fluoranthene	0	0	0	0	0	0	0	0	0	0	0	0
Benzo(a)pyrene	0	0	0	0	0	0	0	0	0	0	0	0
Indeno(1,2,3-c,d)pyrene	0	0	0	0	0	0	0	0	0	0	0	0
Dibenz(a,h)anthracene	0	0	0	0	0	0	0	0	0	0	0	0
Benzo(g,h,i)perylene	0	0	0	0	0	0	0	0	0	0	0	0

The use of a multiplication factor however, meant that there were further errors incurred. This would be greatly improved by (1) automating the extraction method (to reduce variation in the method), (2) using an autosampler for injecting the sample into the GC-MS, (3) further sample repetition and (4) extracting larger quantities of oil.

Unfortunately all such measures were not possible at the time of the investigation. Nevertheless, due to the absence of quantifiable PAHs, it was clear that the majority of PAHs present in the oil were likely to be the methylated species identified in Table 6.7. Alternatively PAHs may have been in such low quantities that they could not be quantified without a further increase in sample volume or sample concentration. More importantly, no single carcinogenic EPA 16 priority PAH was found in large quantities in the oils. Benzo[a]anthracene was found only in oil 8, so contamination of the oil 8 sample could not be ruled out. However, the higher level of mutagenicity observed in the Ames test for oil 8 (Section 5.5.2) may be explained by the presence of benzo[a]anthracene.

From comparison of Table 6.10 with Table 6.7 it can be seen that not all of the EPA 16 priority PAHs identified in the TIC spectra were quantified in the SIC and vice versa. An example of this is benzo[a]anthracene which was identified with SIC and not TIC, or chrysene which was identified in some of the oils with TIC but was not quantified in SIC. Benzo[a]anthracene could not be identified without SIC due to the overlapping of peaks with a similar retention time. Chrysene concentration was not sufficient for quantification, but the peak was visible in the TIC for identification.

A further example of this is benzo[k]fluoranthene which was identified by SIC but which was not quantified due to lack of sensitivity in the GCMS. The possible presence of these carcinogenic PAHs such as benzo[a]anthracene and benzo[k]fluoranthene, in addition to dimethyl benzo[c]phenanthrene, methyl chrysene and dibenzothiophene mutagenicity (Section 6.4.2) may cause the mutagenicity observed in oil extracts with S-9 (Section 5.5.2). In addition, there may be other PAHs in the oil that were not identified in the TIC due to the number of overlapping peaks. Such PAHs may only be identified using SIC if standards of the compound were run

to determine retention time and molecular mass. As the compounds were unknown, this would have been an expensive trial and error process and was therefore not performed.

6.6 EFFECTS OF AGEING ON PAHS IN OIL

6.6.1 Qualitative Variations of PAH in Aged Oils

To determine changes in transformer oils with ageing the TIC spectra of ALT 0, ALT 1, ALT 2 and ALT 3 were compared. This was performed in the same way as for oils 1 to 12, as it had been found that the area of a TIC could be used to evaluate PAH content (Section 6.3). The TIC chromatogram (Figure 6.7) showed that ALT 0 oil had the largest TIC area, which suggested that it had the greatest PAH content. It was also clear from the TIC that ALT 3 had the smallest total area suggesting that area (and therefore PAH content) decreases with ageing. This was investigated further by repeating the extraction of each oil in triplicate and measuring the total area of the TIC of each repeat. This ensured that the results seen in Figure 6.7 were not affected by inefficiencies within the extraction process.

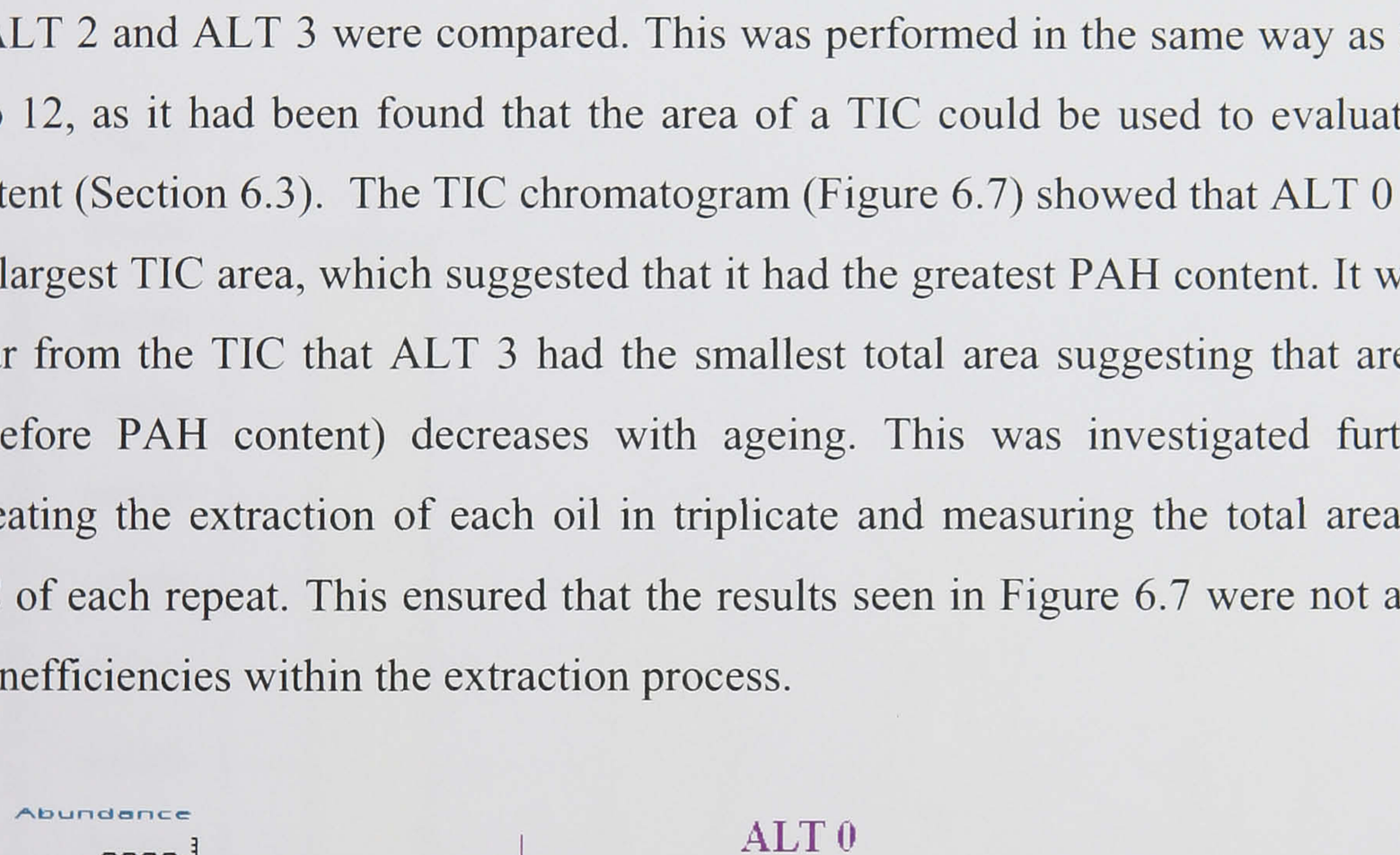


FIGURE 6.7. The TIC of oils ALT 0, ALT 1, ALT 2 and ALT 3.

Figure 6.8 shows that the area of the TIC (and therefore possibly PAH content) did decrease by almost half after 1 week of artificial ageing. This increased on average by 20% after two weeks, although the %CV of 30% suggested that this was not conclusive. The total area of the ALT 3 TIC decreased again to a similar level as ALT 1. The increase in %CV after ageing from 5.5% for ALT 0 to 24-30% (ALT1, ALT 2 and ALT 3) may be explained by the fact that the aged oils contained a high level of particulates produced during oxidation. Although efforts were made to decant the oil from the particulates, it was possible that particulates were still interfering with the extraction, and made the extraction less repeatable.

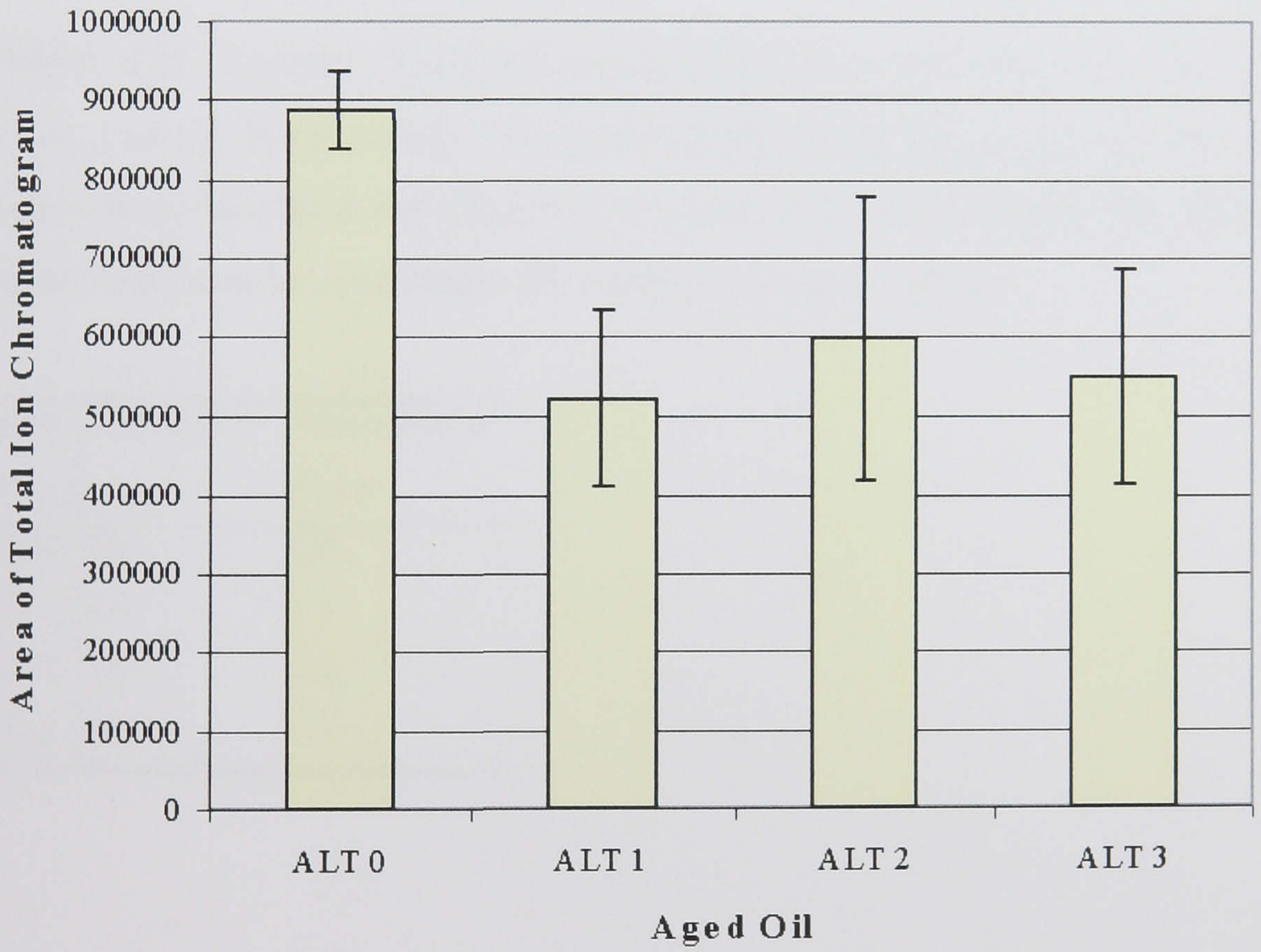


FIGURE 6.8. Area of the total ion chromatogram for aged oils (n = 3). %CV ranges from 5.5-30%.

6.6.2 Quantitative Variations of PAH in Aged Oils

The EPA 16 priority PAHs naturally occurring in the oil were quantified before and after ageing to determine changes in PAH composition. The quantification was performed at the same time as quantification of oils 1 to 12, so the calibration data in Table 6.8 applies here. Multiplication factors of internal standards of deuterated chrysene were used in the same way as in Section 6.5 and the multiplication factors are given in Table 6.11. The figures in Table 6.12 have been multiplied to determine the amount of each PAH if the extraction had been 100% efficient.

TABLE 6.11. Amount of internal standard recovered after injection with oil extracts of oils 1 to 12. Each extract was spiked with 1.8 μg ml^{-1} deuterated chrysene after extraction to determine the efficiency of injection into the GCMS. The multiplication factors were used to compensate for inefficient sample injection.

Aged Oil	*Chrysene-D*	*Multiplication factor*
ALT 0	1.8	1.0
ALT 1	1.8	1.0
ALT 2	1.8	1.0
ALT 3	1.5	1.2

Of the EPA 16 priority PAHs determined with SIC only phenanthrene, fluoranthene and pyrene were found to be present in the oil. The results in Table 6.12 suggested that there was an overall increase in oil PAH content with ageing. Phenanthrene showed a decrease with 1 week of ageing (by 0.1 μg mL^{-1}) which then increased after 2 weeks (by 0.25 μg mL^{-1}) and slightly decreased again after 3 weeks (by 0.02 μg mL^{-1}). Fluoranthene increased after 2 weeks (by 0.2 μg mL^{-1}) and remained at a similar level after 3 weeks.

Finally, pyrene decreased after 1 week of ageing by 0.145 μg mL^{-1} followed by an increase to a greater level than was originally present by three weeks (increase of 0.43 μg mL^{-1}). The overall results for ageing therefore showed a general increase in these three PAHs with ageing. This did not correlate with the finding in Figure 6.8, which suggested that PAH content decreased with age. However, it was postulated that the TIC estimation of total error may have been less accurate with aged oils due to the accumulation of debris and particulates that occur with use. Results are further discussed in Section 7.6.4.

TABLE 6.12. The amount of the EPA 16 priority PAHs found in 100 μL of aged oils. The results have been subject to the multiplication factors in Table 6.11 and multiplied to determine the amount of each PAH if the extraction had been 100% efficient (using extraction efficiencies in Table 4.4).

PAHs	*ALT 0 (μg)*	*ALT 1 (μg)*	*ALT 2 (μg)*	*ALT 3 (μg)*
Naphthalene	0	0	0	0
Acenaphthylene	0	0	0	0
Acenaphthene	0	0	0	0
Fluorene	0	0	0	0
Phenanthrene	0.27	0.16	0.41	0.39
Anthracene	0	0	0	0
Fluoranthene	0.13	0.13	0.35	0.30
Pyrene	0.38	0.24	0.44	0.81
Benzo(a)anthracene	0	0	0	0
Chrysene	0	0	0	0
Benzo(b)fluoranthene	0	0	0	0
Benzo(k)fluoranthene	0	0	0	0
Benzo(a)pyrene	0	0	0	0
Indeno(1,2,3-c,d)pyrene	0	0	0	0
Dibenz(a,h)anthracene	0	0	0	0
Benzo(g,h,i)perylene	0	0	0	0

A large range of PAHs at a higher concentration in the aged oil would have allowed a more accurate monitoring of PAH content with GC-MS. A more appropriate method of determining PAH change with ageing would be to spike the oil with a large quantity of each EPA 16 priority PAH before ageing and determine changes after extraction with the C18/Silica/Isolute PAH HC extraction method. This was not possible at the time of the investigation. Nevertheless, the results suggested that PAH content does change with age and that individual PAHs may be affected in different ways.

6.7 USE OF C18 SILICA ISOLUTE PAH HC EXTRACTION WITH NON-TRANSFORMER OILS

As the C18/Si/Isolute extraction was successfully used with transformer oil, it was possible that other oils could be extracted for PAH identification. A number of oils were extracted, including petrol and red diesel, both thought to contain higher levels of PAH, and edible oils such as Olive oil and Sunflower oil, which it was hoped, contain little or no PAH.

Figure 6.9 shows the TIC of Diesel. The PAHs found are labelled. It was clear that diesel had a high PAH content, and the extraction method produced a clean extract. The Spectrum in Figure 6.9 is only shown at up to 14 minutes as there were no PAHs of later retention time found. The red diesel, like transformer oils contained many fluorene and phenanthrene derivatives with the addition of anthracene and pyrenes, which were not as prevalent in transformer oil.

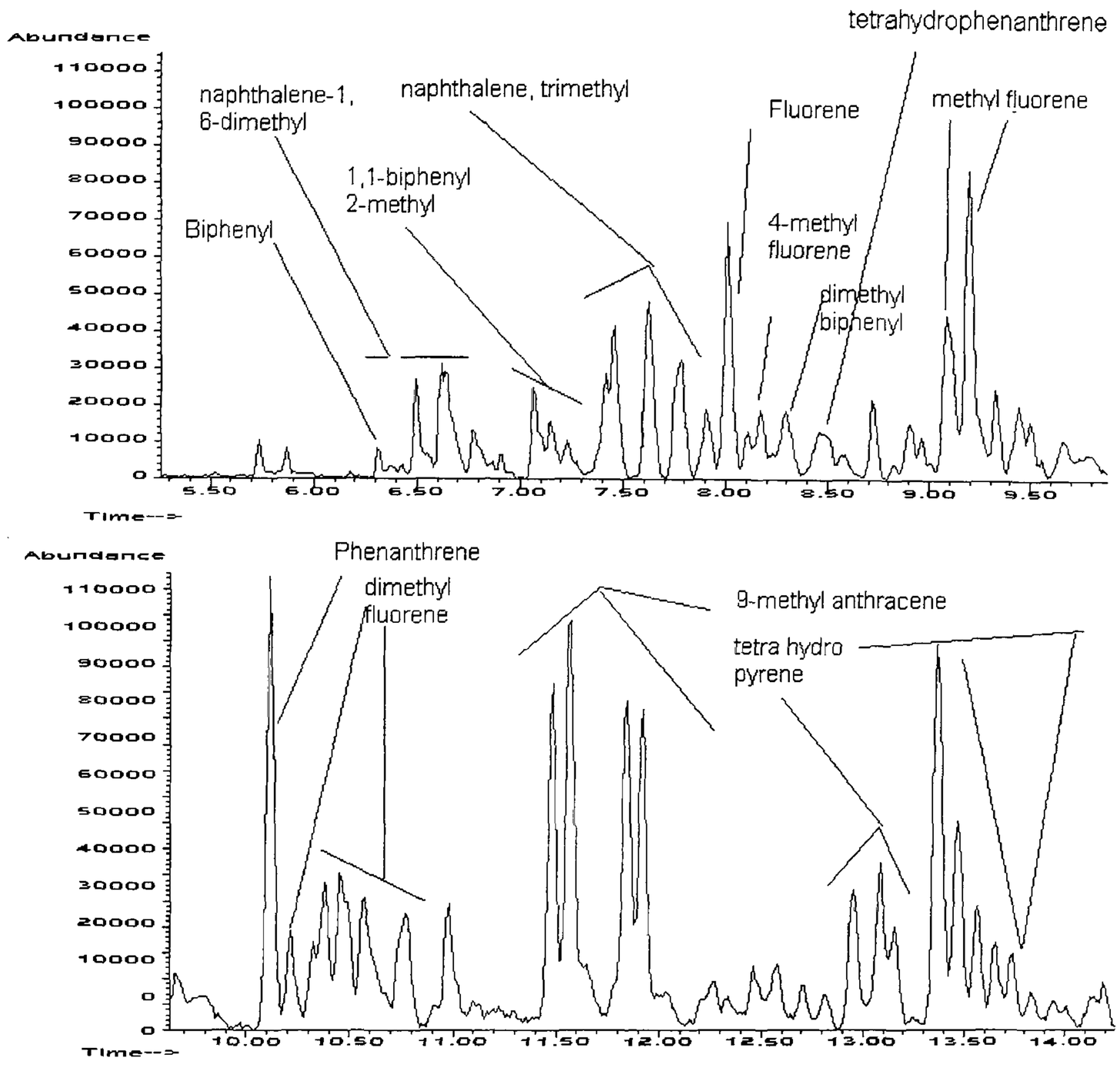


FIGURE 6.9. Close up of diesel extracted by C18 Silica Isolute PAH HC method.

Naphthalenes were also identifiable in red diesel, but were not observed in transformer oils, although the mineral insulating oil fingerprinting technique indicated their presence. This showed that the C18/Silica/Isolute PAH HC method was capable of extracting naphthalenes, although it was not understood why it did not extract them from the transformer oil. It was possible that the extraction of naphthalene was inefficient and that there was not enough of each naphthalene to ensure detectable amounts were extracted in transformer oil. Most importantly, no large carcinogenic PAHs of the EPA 16 priority PAHs were identified.

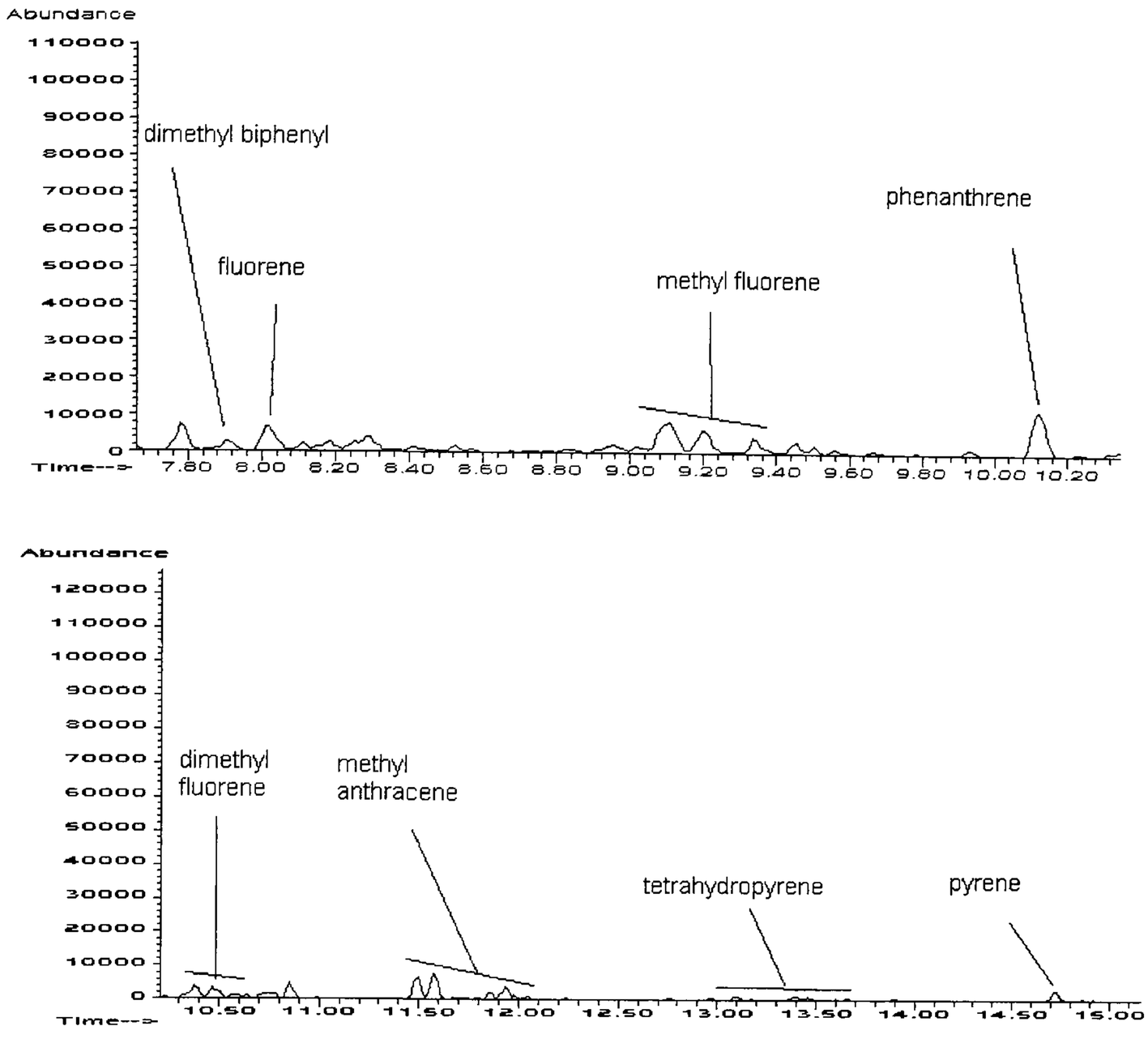
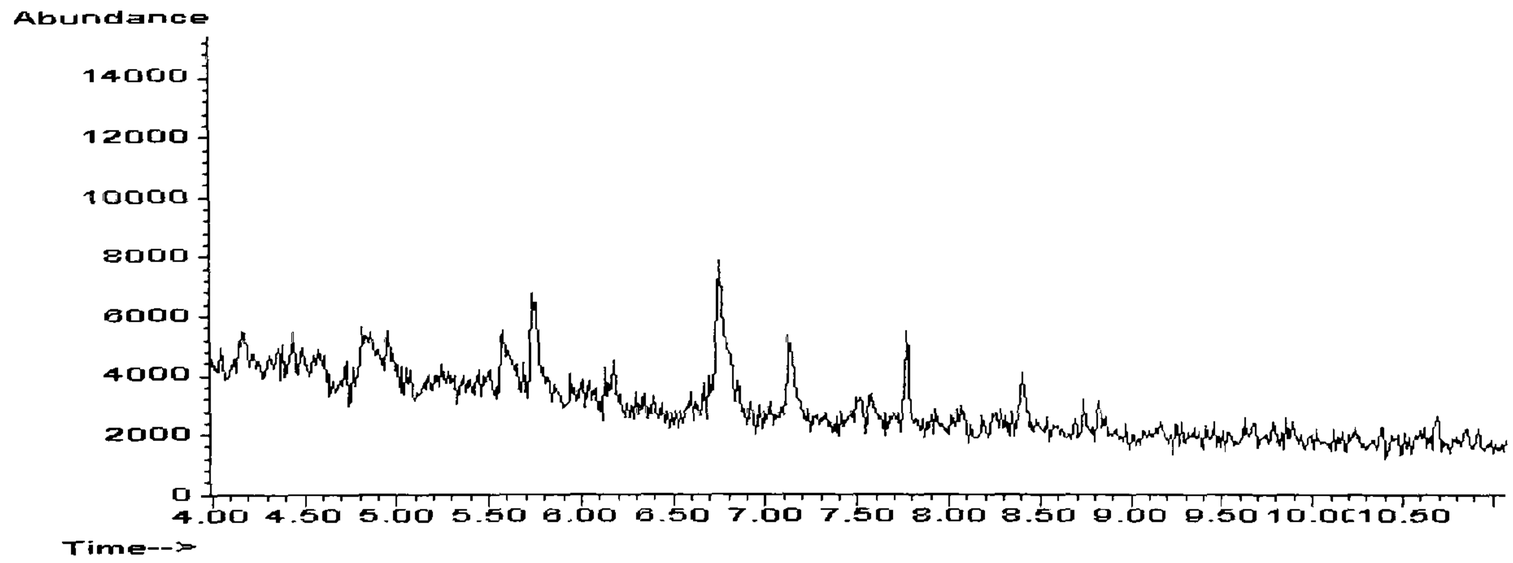


FIGURE 6.10. Close up of petrol extracted by C18/Silica/Isolute PAH HC method.

Figure 6.10 shows the TIC of petrol. It was clear that the petrol did not contain as much PAH as the diesel, but once again an abundance of fluorene and phenanthrenes are found. No carcinogenic EPA 16 priority PAHs were identified. Figure 6.11 and 6.12 respectively, show the TIC of C18/Silica/Isolute extracts of olive oil and sunflower oil. No PAHs of any type were found in any of these oils.



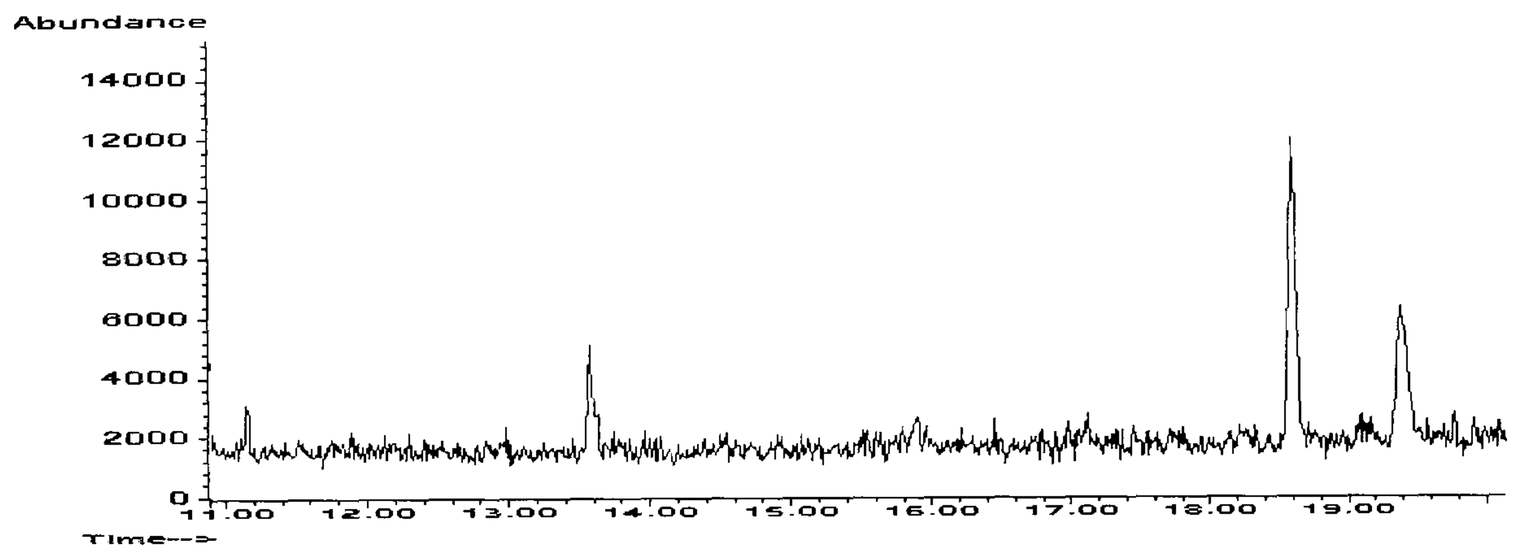


FIGURE 6.11. Close up of Sunflower Oil extracted by C18/Silica/Isolute PAH HC method.

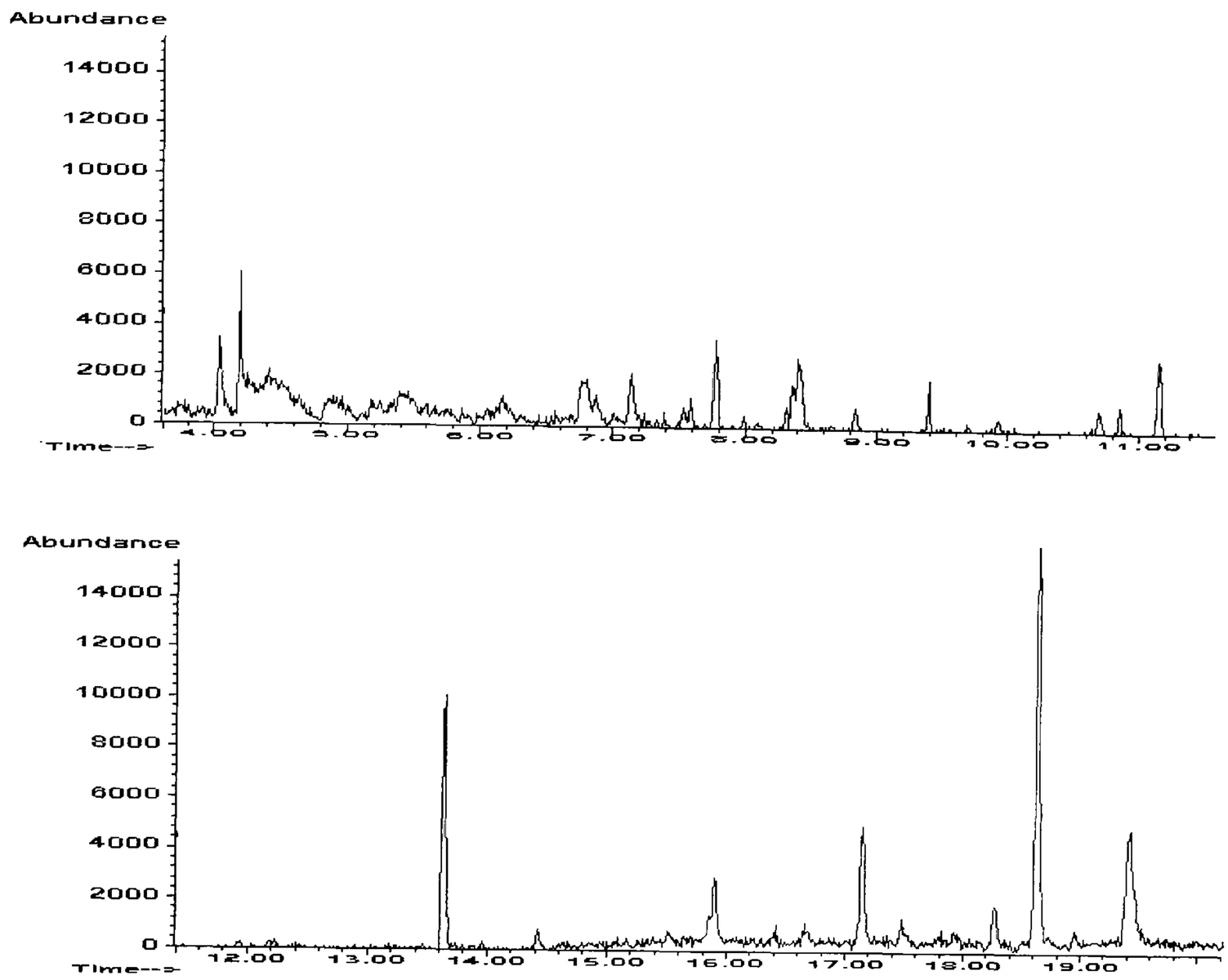


FIGURE 6.12. Close up of Olive Oil extracted by C18/Silica/Isolute PAH HC method.

6.8 CONCLUSIONS

The C18/Silica/Isolute PAH HC extractions with GC-MS analysis clearly had benefits not present for the IP 346 % w/w method or immunoassay detection. GC-MS analysis of IP346 samples was inconclusive for PAH identification or quantification, due to the complexity of the sample and immunoassay buffer environments were not compatible with GC-MS. Using the C18/Silica/Isolute PAH HC extracts to determine total PAH content would provide a simply, fast, less hazardous and less expensive method that fulfils the criteria of IP 346 estimation but that can also be used to analyse all aspects of PAH content.

CHAPTER 7.0

DISCUSSION AND FURTHER WORK

7.1 INTRODUCTION

PAHs are natural components of the transformer oils that reduce the rate of oil breakdown and therefore prolong the life of electrical power transformers. Some of the larger PAHs have been found to be carcinogenic however, and for this reason the National Grid Company Plc are concerned that PAHs may be of risk to the health of employees exposed to oil. Legislation produced by CONCAWE, for the labelling of oils states that oil with an polyaromatic content of over 3% w/w according to IP 346 gravimetric quantification, must be labelled as hazardous. Oils with an IP 346 % w/w values over 3% are of risk according to skin painting data (Stang, 1993; 1999; 2000). Although PAHs are thought to be the main source of oil mutagenicity (McKee *et al*, 1989) the IP 346 overestimate of polyaromatic content (Section 1.1.3.1) so cannot be related to PAH risk alone. Therefore the PAH content, rather than the aromatic content must be identified and analysed for mutagenicity to determine if PAHs are the main risk from oils, or if the aromatic IP 346 fraction contains other mutagens.

Much analysis has been performed to determine PAHs in mineral oils but is complicated by the fact that each oil is composed differently. Mineral oils are refined to different extents according to their uses. Refining processes may be categorised as unrefined, slightly refined and heavily refined. Although it may be safe to assume that unrefined oils are more dangerous than slightly refined oils and that heavy refining reduces mutagenicity (IARC, 2002) it cannot be assumed that all oils within the same class of refinement have broadly the same PAH composition or mutagenic threat.

Even within a specific application, such as use in transformers, PAH content will vary greatly according to the origins of the oil (illustrated by IP 346 % w/w values in Table 2.1). Each oil therefore, must be analysed and assessed individually. For this reason, although a number of extraction methods were taken from the literature, the results of other work could not be directly compared to those in this study as the oils were so different in composition and usage. No data was found in the literature for the

particular transformer oils used in this work, so no indication of possible outcome could be assessed before the work was completed.

7.1.1 Aims and Approaches of the Work

The main aim of this work was to identify PAH composition in the oil and demonstrate possible mutagenic risks from PAHs to National Grid Company Plc employees. An outline of the work progression is given in Figure 7.1. This would involve providing evidence of total oil mutagenicity to establish if PAHs are the main source of mutagenic risk. This would indicate if efforts should be made to remove PAHs from oil and replace them with an artificial inhibitor that may itself pose a threat to health (Section 1.1.3). It would also determine whether the current labelling legislation was sufficient to protect the health of employees. In addition, it would help identify environmental concerns about PAH contamination from oil leakage, which is becoming an important issue particularly in the UK and USA (EPA, 2002).

As PAH content of all mineral oils vary greatly, 14 transformer oils commonly used by the National Grid Company Plc were tested. When resources were limited, 4 of these oils were selected for in-depth testing based on their widely differing aromatic content (IP 346 % w/w values) so that general trends could still be observed. Oil 8 contained the greatest amount of PAH, oil 4 the least and Nytro-10GBN contained an intermediate amount and was widely used by the National Grid Company Plc. White oil was also used to give a background "blank" reading as it was highly refined and thought to contain little or no PAHs.

The Ames test is an effective method of testing for mutagenicity and is a screening method for detecting possible carcinogens without animal testing. The test remains one of the cheapest and effective ways of testing mutagenicity. It has been modified to suit its application to oils and involves the extraction of the aromatic portion of the oil as well as the increased use of S-9 activation enzymes (Blackburn *et al.*, 1986). This

modification made the Ames test the obvious choice for testing the mutagenicity of transformer oils.

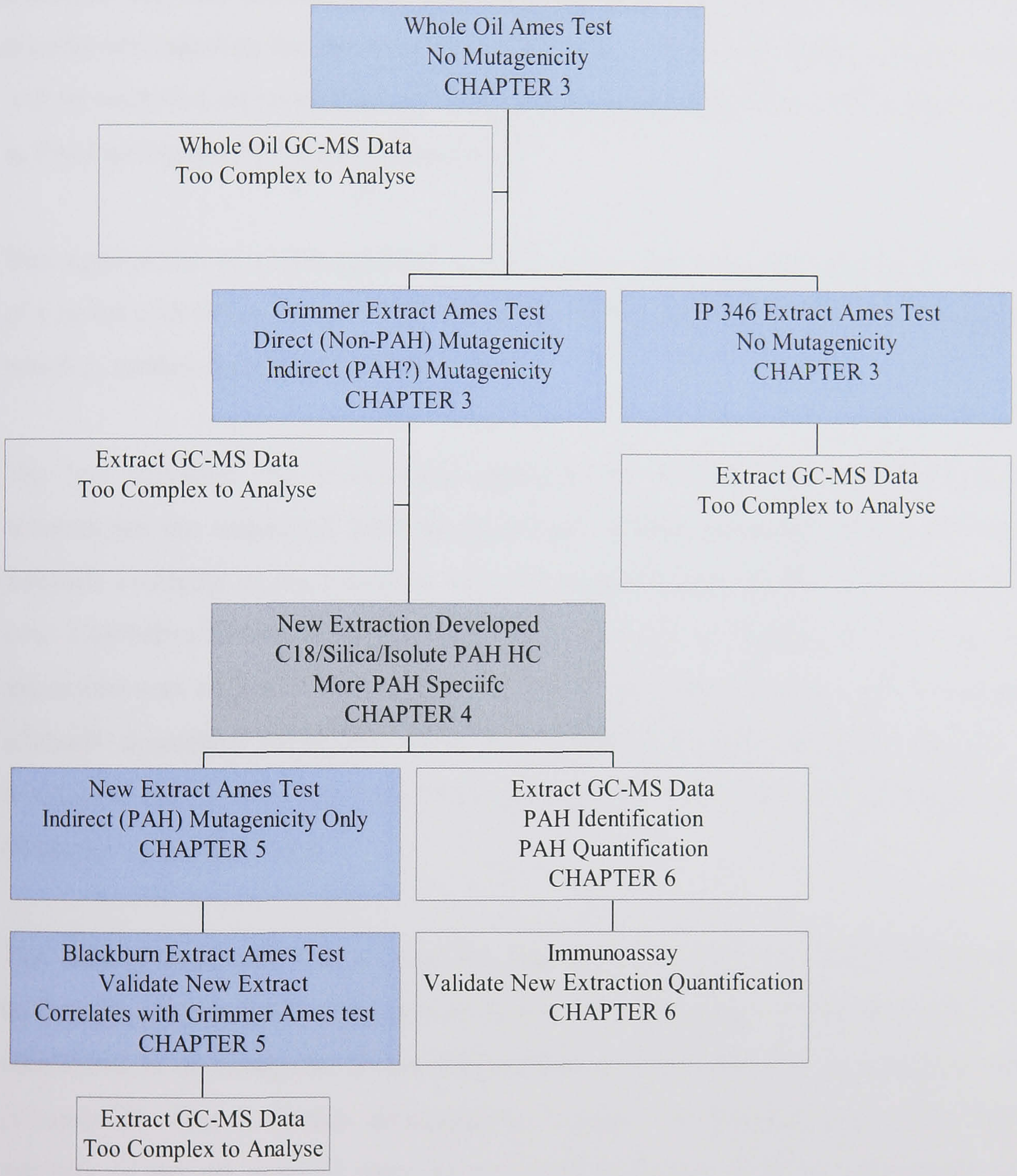


FIGURE 7.1. Outline of work progression throughout this thesis to determine the mutagenicity of PAHs in transformer oil.

One difficulty in PAH mutagenicity testing is that different methods of extraction yield different results (Stang, 1993; 1999). This discrepancy is compounded by the fact that the extracts used to measure mutagenicity are never analysed to identify PAHs, as they are too complex. This means that the presence of a mutagen is never directly attributed to the presence of a PAH. For this reason, whilst current methods will be analysed, an extraction method that can be used for both PAH analysis as well as PAH mutagenicity tests was developed.

Two approaches to assessing PAH content were used based around the development of a novel C18/Silica/Isolute PAH HC extraction method for PAHs in oil (Chapter 4), which is further discussed in Section 7.3.

The first approach was to measure mutagenicity directly using the Ames test and demonstrate the impact of PAH mutagenicity on total oil mutagenicity. The current methods available in the literature were investigated prior to the development of the new C18/Silica/Isolute PAH HC extraction method to determine if an improved extraction was required (Chapter 3). When it was found that the current extraction methods contained mutagens other than PAHs, the new extraction method was developed and used to establish PAH mutagenicity only (Chapter 5). This is further discussed in Section 7.4.2.

The second approach was to use the new C18/Silica/Isolute PAH HC extraction method to analyse PAH composition of oil. This determined if analysis allowed the identification of mutagenic threat without the need for complex mutagenicity testing (Chapter 6). This is further discussed in Section 7.6. Different aspects of GC-MS analysis of the oil extracts were investigated including direct quantification of the EPA 16 priority PAHs, identification of other PAHs (particularly mutagenic ones) and an estimation of total PAH content by integrating the whole GC chromatogram of each oil. This method was compared to other methods already available, namely IP 346 and immunoassay methods.

Initial concerns with the work originated from the complexity of the oil matrix. Oil is composed of many different compounds, and it was possible that transformer oil contained a variety of both mutagenic and toxic compounds. Toxic effects would reduce the amount of *Salmonella typhimurium* present and could cause a reduction in revertant number that would lead to false results. Additionally, there were concerns that some oil components may be antagonistic to mutagenic PAHs, once again producing false Ames test results. For these reasons, inhibition and toxicity affects on the Ames test were also investigated and are discussed further in Section 7.5.1 and 7.5.2 respectively.

Finally, artificially aged oils were briefly analysed to see if PAH composition and therefore mutagenicity changed with in-service usage. This would have great implications on the electricity industry, as it would determine if monitoring PAH levels during the lifetime of the oil was required or if testing before use was sufficient. This is further discussed in Section 7.2.5, 7.4.3 and 7.6.4.

7.2 MUTAGENICITY OF PAHS IN OIL

The oil was tested as a whole, and then extracted using methods reported in the literature (Chapter 3) to determine the PAH contribution to oil mutagenicity.

7.2.1 Ames Testing of EPA 16 PAH Standard

Testing some of the EPA 16 priority PAHs separately established the *S. typhimurium* strain's (TA98) sensitivity to PAHs (Section 3.2.1.1). The findings agreed with the literature showing that the *S. typhimurium* strain was behaving as expected and was appropriate for use with PAHs (Hermann, 1980). Chrysene, was the only PAH of those tested that did not show a clear positive or negative response. There is contention in the literature as to whether *S. typhimurium* shows mutagenicity in the presence of chrysene. For example, Basler *et al.* (1977) found no mutagenicity, while McCann *et al.* (1975) identified mutagenicity. The intermediate responses observed

here supported these inconsistencies in the literature, and illustrated the limitations of the Ames test for the analyte. If such an inconclusive result was obtained from the oil samples, a further mutagenic test such as Mutatox™ would be required.

Testing was then performed on a mixture of the EPA 16 priority PAHs to determine the mutagenicity of a mixture of these compounds (Section 3.2.1.2). It was found that antagonistic effects were present and toxicity effects may be acting to mask mutagenicity. This would lead to positive results being mistaken for negative results and illustrated the requirement for toxicity testing in conjunction with mutagenicity testing.

7.2.2 Ames Testing of Whole Oil

Although modifications have been made to improve the Ames test for oil analysis by extracting the aromatic fraction, the initial concern was to test the oil in its unmodified form. Extracting the aromatic fraction of the oil as described by Blackburn *et al.* (1986) meant that any mutagens present in the remainder of the oil were not accounted for. In addition, it was interesting to see if the whole oil mutagenicity differed from the mutagenicity of the aromatic extract. It was found that the whole oil, whether added directly, or made more compatible to the aqueous environment with Tween 80, showed no mutagenic effects (Section 3.3.1 and 3.3.2).

There were three possible causes for this:

1. The oil was not mutagenic. This could be resolved by testing the aromatic fraction of the oil (discussed in Section. 7.2.3).
2. The oil environment was not compatible with the Ames test, and inhibited either the growth of *S. typhimurium*, or affected the enzymatic properties of the S-9 fraction (Watson *et al.*, 1985). The oil matrix was shown to affect *S. typhimurium* in particular (Table 3.6). It was considered that this effect may be overcome by extracting the oil before testing.

3. Due to the many synergistic and antagonistic effects occurring within the oil, the oil matrix may trap or deactivate mutagens (Watson *et al.,* 1985). This was tested by spiking the oil with a known mutagen (benzo[a]pyrene or 2-amino anthracene) and observing any reductions in reversion number. This inhibition test was performed for all the oil extracts used in this work and is therefore discussed collectively (Section 7.5.1).

7.2.3 Ames Testing of IP 346 and Grimmer Extracts at 10% S-9

The mutagenicity of an oil is best determined by extracting the aromatic fraction of the oil (Blackburn *et al.,* 1986). The amount of oil extracted and tested was limited by the end user's health issues arising from using large volumes of solvent and by the amount of extract that was soluble in the minimum solvent volume required for the Ames test. For this reason no more than 5 g of oil was extracted (Section 1.4.1.6). The method used by Blackburn *et al.* (1986) includes the use of an 80% v/v S-9 mix and a liquid-liquid extraction (LLE) step, using DMSO and cyclohexane.

The extraction worked on the same principle as the IP 346 % w/w method used to estimate polyaromatic content (Section 1.5.2). As IP 346 is an industry standard LLE method, the use of LLE was deemed appropriate for measuring PAH mutagenicity in oil. As with the IP 346 % w/w method however, the Blackburn extracts uses DMSO to extract polyaromatics (Section 1.1.3.1) and therefore may extract simple aromatic and other polar components in addition (Stang, 1993, 1999). This suggested that mutagens other that PAHs may be extracted by these methods.

As well as the IP 346 % w/w method and the Blackburn method, the LLE method used by Grimmer *et al.* (1981) was also employed to determine if the type of extraction method altered the mutagenicity measured. The extractions, although using the same solvents, contained different numbers of washing steps, which may affect the efficiency of extraction. The IP 346 % w/w method, when used as a gravimetric

estimation of polyaromatic content was believed to correlate well with skin painting data, (Stang, 1993; 1999; 2000) so was used with the Ames test initially. The Grimmer method was also investigated, as it was much simpler to perform (Section 3.4.1).

As the Blackburn method was the most popular method for use with the Ames test for oils (Brooks *et al.,* 1995; Granella *et al.,* 1995), it was not tested until the C18/Silica/Isolute PAH HC extraction had been developed. This allowed a direct comparison and validation of the new method with an established method of measuring PAH mutagenicity (Section 5.2) from oils and is discussed in Section 7.4.1.

The dose is reported as milligrams of oil extracted rather than milligrams of oil extract and is plotted against the mean number of revertants per plate. This allowed a simpler comparison of whole oil mutagenicity results with oil extract results.

Oil 8, oil 4 and Nytro-10GBN were extracted using the IP 346 % w/w method and the Grimmer method to provide data on a range of oils, having differing aromatic contents. The extracts were Ames tested over 5 consecutive doses (0.5-500 mg of oil per plate) to determine the best dose with which to test further extracts. Only 10% v/v S-9 was used for the initial testing since an 80% v/v loading (Blackburn *et al.,* 1984) would be prohibitively expensive to the end user for routine sample analysis over 5 doses.

The trends in Ames test data from all aromatic extracts of oil 8, Nytro-10GBN, 4 and white oil are compiled in Table 7.2 in Section 7.4.2.1 for comparison to the new C18/Silica/Isolute PAH HC extraction, but Grimmer and IP 346 extracts are discussed here initially.

7.2.3.1 Grimmer Method

The Grimmer extracts produced a mutagenic response for white oil, oil 8, 4 and Nytro-10GBN without S-9, which suggested that a direct mutagen (mutagenic without S-9 activation) was present (Section 3.4.1 and Table 7.2). Direct mutagens present in mineral oil were also acknowledged by Granella *et al.,* (1995). The reversion number was high at ~1000 revertants plate^{-1} at an oil extract dose of 500 mg plate^{-1}. As these mutagens were direct, the mutagenesis could not be due to PAHs, as PAHs must be activated into their mutagenic form by S-9 enzymes. This suggested that there were other unknown mutagens in the oil that could threaten the health of oil users. Removing PAHs from the oil would only reduce the mutagenic threat therefore, unless this direct mutagenic threat was also identified and removed. The direct mutagen was believed to be an aromatic compound, known to be extracted with polyaromatic compounds in liquid-liquid extractions (Stang, 1993; 1999).

For the Grimmer extracts with S-9, mutagenicity was slightly greater than that observed without S-9 for Nytro-10GBN suggesting an accumulation of mutagenicity from direct and indirect mutagens. However, no increase in mutagenicity was observed for white oil, oil 8 or oil 4 with S-9. For oil 4 and white oil, the absence of mutagenicity was thought to be due to insufficient PAHs in the oil. However, as oil 8 was found to have the greatest PAH content (Section 6.2.3, 6.3.1) the majority of which were later identified as non-mutagenic PAHs (Section 6.4.2), it was postulated that competitive or non-competitive inhibition of the S-9 enzymes from non-mutagenic oil PAHs was the cause of reduced mutagenicity. This was later validated and is discussed further in Section 7.5.1.

Nevertheless the results from using 10% v/v S-9 were used to draw a number of conclusions that would be useful in further testing. The results indicated that the largest dose used (500 mg plate^{-1}) was required to determine mutagenicity. It also demonstrated that the Grimmer method was better than the IP 346 method at revealing mutagenic content. Most significantly, the results revealed direct mutagen(s) were

present indicating that indirect mutagens such as PAHs were not the only source of mutagenicity.

7.2.3.2 IP 346 Method

It was found that the IP 346 extracts for all the oils were not mutagenic with or without S-9 (Section 3.4.1 and Table 7.2). This suggested that mutagens were not present in the aromatic fraction in sufficient quantities to elicit a positive Ames test response. Alternatively lack of mutagenicity could be caused by an insufficient amount of activation enzymes. It was possible that there were non-mutagenic PAHs competing for the active sites of the enzymes and therefore reducing mutagenicity.

Although no mutagenicity was observed with IP 346 extracts, the use of IP 346 % w/w values as a measure of total aromatic content of the oil (as opposed to PAH content only), has been justified by the presence of direct mutagens in the aromatic fraction. If only the PAH content had been extracted, the full mutagenic nature of the oil would not have been established. These findings indicated that extracting the total aromatic fraction of the oils is potentially the most viable way to measure oil mutagenicity. However, concluding that the mutagenicity in the aromatic content is due to the polyaromatic content (Grimmer, 1983; Järvholm and Easton, 1990; Brooks *et al.,* 1995; Granella *et al.,* 1995) is not justified for the oils tested in this work due to the presence of additional direct mutagens.

As the Grimmer extracts revealed mutagenicity at 10% S-9, Grimmer extracts of white oil, oil 4, oil 8 and Nytro-10GBN were repeated with 80% v/v S-9 to determine if an increase in activation enzymes affected the results (Section 3.4.2).

7.2.4 Ames Testing of Grimmer Extracts at 80% S-9

As the Grimmer method appeared superior to the IP 346 method with regard to mutagenicity analysis, extracts were tested with elevated levels of S-9 (80% v/v) over 3 doses for oil 8, 4, Nytro-10GBN and white oil (Section 3.4.2 and Table 7.2). The increase to 80% v/v in S-9 enzymes produced ~200-500 more revertants with oil 8 than for oil 4 and Nytro-10GBN. The number of revertants for oil 8, on the addition of 80% v/v S-9 had doubled compared to the number of revertants without S-9. This doubling in reversion number clearly showed that indirect mutagens (possibly PAHs) were present.

As the number of revertants had increased for oil 8 at elevated levels of S-9, it was believed that the same outcome may occur for oil 4 and Nytro-10GBN. Revertant number did not significantly change for oil 4 on the addition of 80% v/v S-9. The same number of revertants was observed with or without S-9, suggesting that oil 4 did not contain a sufficient amount of PAH to induce a positive response. The same response was observed for white oil showing that the 'blank' oil contained direct mutagens only. These results showed a similar trend to those in Granella *et al.,* (1991, 1995) where liquid-liquid extracted oils with a low PAH content (in this case recycled oils) showed only direct mutagenesis while those with a high PAH content showed both direct and indirect mutagenicity.

An increase of 200 revertants (24%) previously observed for Nytro-10GBN with 10% v/v S-9, increased to 300 (35%) with 80% v/v S-9. This increase was not considered significant and suggested that there was a sufficient enzyme loading in 10% v/v S-9 to activate the majority of mutagenic PAHs in Nytro-10GBN.

The IP 346 % w/w quantification of polyaromatic content (Table 2.1) showed oil 8 to have a value of 8.9% w/w, while oil 4 contained 0-1% w/w and Nytro-10GBN contained around 1.75-2.64% w/w. If IP 346 correlated with Ames test data as stated by the oil manufacturer, Nynas (Stang, 1993; 2000), oil 8 should have a much greater

mutagenic response with S-9 than the other oils. In turn Nytro-10GBN should have given a greater response than oil 4 and white oil. Therefore, the Ames test results of the Grimmer extracts correlated with IP 346 % w/w data as white oil and oil 4 gave no indirect mutagenicity, while Nytro-10GBN gave a 35% increase in revertant number and oil 8 a 50% increase in the presence of activation enzymes.

Identification of the individual indirect mutagens was not attempted using these extraction methods however, due to the complexity of the oil extracts as shown by the GC-MS total ion chromatograms (TIC) in Section 3.4.1.1.

7.2.5 Ames Testing of Aged Oil Grimmer Extracts at 80% S-9

It has been reported in the literature that PAH levels can increase over time in oils subjected to high temperatures during there working life (Moret and Conte, 2000; Wang *et al.,* 2000; Wong and Wang, 2001). As transformer oil will be subject to oxidation and heat during its lifetime, PAH composition must be monitored to determine the build up of PAHs (Section 7.6.4) and changes in mutagenicity must be assessed.

Aged oils were also investigated using the Grimmer method (Section 3.5). It was found that once again mutagenicity was present without S-9 for all oils, but no change in revertant number was observed with ageing. In addition, the increase in reversion number (~14-20%) on the addition of S-9 was not as great as that observed for Nytro-10GBN or oil 8. Nevertheless it was possible that such a consistent increase was due to indirect mutagens.

7.2.6 Ames Testing of Solid Phase Extracts

Solid phase extraction (SPE) was performed as an alternative method to LLE (Section 3.6). It was postulated that the SPE would produce a cleaner extract that could be analysed by GC-MS to identify the mutagens responsible. It was also believed that by

fractionating the extract during elution, the complexity of the extract would be reduced and less S-9 (10% v/v) would be required during Ames testing.

Fraction 1 of oil 4 proved mutagenic without S-9 (as with the Grimmer and Blackburn extracts). However, the number of revertants increased by over 200 (4.5 fold) on the addition of 10% v/v S-9 suggesting the additional presence of an indirect mutagen, which was not observed with the Grimmer or Blackburn extracts. Nytro-10GBN also showed mutagenicity in fraction 1 without S-9 and revertants increased by over 100 (1.6 fold) on the addition of S-9 (refer to Table 7.1 for summary of results). These results suggested that oil 4 contained more indirect mutagens than Nytro-10GBN, but this result was not conclusive as SPE was unable to remove some of the aliphatic components of the oil, contributing to a lack of precision (%CV up to 42%) in the Ames test results.

TABLE 7.1. Summary of Ames test results for SPE extracts. The mean number of revertants is shown.

Oil	*SPE Fraction 1 (10% S-9)*		*SPE Fraction 2 (10% S-9)*	
	(-)S-9	*(+)S-9*	*(-)S-9*	*(+)S-9*
4	83	370	35	29
Nytro-10GBN	14	221	35	32
8	24	252	177	217

Although a lack of precision affected results, the fractionation of the aromatic extract showed that the mutagens were probably resident within the less polar fraction (fraction 1). No mutagenicity was found in fraction 2 of oil 4 and Nytro-10GBN with or without S-9. This suggested that approximately half of the fraction extracted by liquid-liquid methods was not mutagenic and should ideally be removed for mutagenicity measurements, as they may cause interference.

Different results were obtained for oil 8. Mutagenicity was only observed for fraction 1 when S-9 was added (increase of 5 fold). The direct mutagens were found in

fraction 2. This indicated that the properties of the indirect mutagens were different enough to allow separation from the direct mutagens, although this extraction method was not effective enough to allow this for all the oils.

The TIC of the two fractions of Nytro-10GBN (figure 3.14) indicated the limitations of this extraction method as SPE failed to produce cleaner extracts than LLE (IP 346 and Grimmer). PAHs and other possible mutagens were therefore not identified.

As LLE benefited from better repeatability due to the removal of aliphatic components, but SPE benefited from fractionation, a combined process was employed (Section 3.7). The results however, indicated no mutagenicity or toxicity, possibly due to PAHs being fractionated between the cyclohexane wash and the two collected fractions, so that no single fraction contained sufficient PAHs to cause a mutagenic effect. The GC-MS TIC scan once again indicated inefficient separation of PAHs from the other aromatic compounds (figure 3.16). A novel approach was therefore required to conclusively determine the mutagenicity solely due to PAHs.

7.3 DEVELOPMENT OF A NEW EXTRACTION METHOD FOR TESTING OIL PAH MUTAGENICITY

Current methods of measuring the mutagenicity of oil, such as that described by Blackburn *et al.* (1984, 1986) produced extracts that were found to contain many interfering components. These extracts although useful in determining overall mutagenicity, could not be used to determine PAH induced mutagenicity. For this reason, a study was initiated based on the development of a new extraction method, with greater specificity to PAHs.

7.3.1 Solid Phase Extraction (SPE)

From primary studies of extraction methods used for oil PAH mutagenicity tests, LLEs were the most popular methods. However, GC-MS analysis of such extracts confirmed the suggestion in the literature (Stang, 1993; 1999) that LLEs extract all aromatics rather than PAHs. SPE was often employed after the LLE process when cleaner extracts were required (Wang *et al.,* 1999; Grimmer *et al.,* 1981). The main benefit of SPE is the ease by which samples can be fractionated, allowing more subtle separation of components with similar properties. SPE was found to be more effective that LLE for the recovery of PAHs by Sargenti *et al.* (1998). Although SPE had not proven successful in previous studies (Section 3.6 and 3.7) it was considered a more appropriate choice for further development as it typically consumes less hazardous solvent and has potential for automation.

Of the many sorbents used in SPE, only silica (SiO_2) was found to be an appropriate sorbent for PAH extraction from the literature. Alumina (Al_2O_3) was suggested as an alternative to SiO_2 (Thurman and Mills, 1998) but proved to be no better at extracting PAHs from oil (Section 4.3.1). Cyanopropyl sorbents was used as a pre-cleaning step before SiO_2 but added no benefits to extraction efficiency (Section 4.3.2). SiO_2 therefore remained the main extraction sorbent on which the rest of the extraction was based. Parameters such as column size, shape, sample volume and solvent type and volume were all varied to optimise this extraction method (Section 4.2.2.1 to 4.2.2.4). It was clear from the complexity of the oil sample that only a multi step extraction with a combination of sorbents would be effective. It was postulated that the most demanding phase of the extraction would be to remove the PAHs from other aromatics due to their similar properties.

7.3.2 New Sorbents

Of the extraction sorbents investigated, two were identified as having the most potential for PAH extraction. The first was a sorbent specifically designed for the extraction of PAHs in soil and water. The Isolute PAH HC sorbent proved to be ineffective with whole oil (Section 4.3.3). However, the sorbent showed promise when used after a clean up stage such as LLE (Section 4.5.1) and gave a clean extract for oil 8 that could be analysed for PAH content with GC-MS. However, the extraction produced no PAH peaks for oil 4 or oil Nytro-10GBN after GC-MS analysis. It was postulated that the aromatic content of the oil affected the extraction efficiency as only the high aromatic content of oil 8 was extracted. A possible improvement was to increase the amount of oil used in the initial LLE, but as the LLE was already time consuming (1-2 hours) and used appreciable volumes of hazardous solvent (a minimum of 100-200 mL), an alternative clean up stage was investigated for use with the Isolute PAH HC sorbent.

The second sorbent identified as having potential for PAH extraction was used in the fingerprinting technique (Wilson and Pahlavanpour, 2000) (Section 4.4.1). The C18 column was utilised to bind the less polar components in the oil while the PAHs were transferred through the column using cyclohexane. This was particularly useful at separating non-polar components that varied only slightly in polarity and that would not have been separated in a normal phase extraction (Section 1.5.3.1).

7.3.3 The C18/Silia/Isolute PAH HC Method

The C18 column was used as a clean-up step before Isolute PAH HC purification (Section 4.5.2) but proved inefficient until a SiO_2 column was added as an extra clean up step, as used in the mineral insulating fingerprint technique (Section 4.5.3). Unlike the fingerprinting technique, adding the SiO_2 column after the C18 column produced the most efficient cleaning. This C18/silica clean up step followed by the Isolute PAH

HC purification proved to be highly effective at PAH extraction, producing a clean GC-MS chromatogram with clearly defined peaks (Section 4.5.4).

One drawback to the use of the Isolute PAH HC extract was the large peak found by the GC-MS at a retention time of 21 minutes (Figure 4.9). This peak was unidentifiable (NIST MS library) but was present whenever the Isolute PAH HC sorbent was used. As the Isolute PAH HC column was the final step in this new extraction, the eluting species could not be extracted without further preparative steps. However, this peak did not interfere with the data gained from the chromatogram and did not affect mutagenicity data (Section 5.5.1) so was not investigated further.

To summarise the extraction procedure (detailed in Section 2.2.9), 100 μL of oil was washed through a 900 mg C18 column with 3 mL cyclohexane onto a 1.2 g silica column which was further washed with 5 mL cyclohexane. PAHs were then eluted in 6 mL acetone and after evaporation reconstituted in 1 mL cyclohexane and added to a 1g Isolute PAH HC column which was washed with 3 mL pentane. The final 6 mL acetone eluent was then quantified using GC-MS according to Section 2.2.6.1.

To test the efficiency of the procedure, a 100μL sample of pure oil was spiked with 2 μg mL^{-1} of each EPA 16 priority PAH and extracted. In determining carcinogenic risk to humans, the larger PAHs were of most interest. They were extracted with efficiencies ranging from 68-84% (Table 4.4 and 4.5) and the chromatogram at later retention times (20 minutes onwards) was clean enough to allow peaks to be visualised in the TIC mode (Figure 4.19). The process was found to operate at this efficiency with a PAH loading of up to 4 μg mL^{-1} of each EPA 16 priority PAH (Section 4.6.3).

Extraction efficiencies of the non- mutagenic PAHs such as fluorene, phenanthrene, anthracene, fluoranthene and pyrene ranged from 21-67%. The volatile PAHs, naphthalene, acenaphthylene and acenaphthene were lost in the process due to concentrating the extract by evaporation. Wang *et al.* (2000) identified a 40% loss of acenaphthene due to such a procedure. These volatile PAHs were not of concern in

this work as they had not been found to have a mutagenic impact (IARC, 2002). Rotary evaporation used instead of evaporation at room temperature increased the amount of volatiles recovered, but was not used as it reduced the recovery of the larger carcinogenic PAHs. The loss was incurred when removing the evaporated extract from the round bottomed flask of the rotary evaporator in a minimal solvent volume (Section 4.6.1).

There were two modes of analysis for the new extract. The first was the investigation of mutagenicity with the Ames test (Chapter 5) which was compared to work performed with extraction methods previously developed (discussed in Section 7.4). The second was the GC-MS analysis of the extracts for PAH identification and quantification which was compared to the fingerprinting technique described by Wilson and Pahlavanpour, (2000), IP 346 data and immunoassay detection (Chapter 6). This is further discussed in Section 7.6.

7.4 AMES TESTING OF NEW EXTRACTION METHOD

The new C18/Silica/Isolute PAH HC extraction method was used to test oil mutagenicity as GC-MS analysis showed the oil extracts to be rich in PAHs. In parallel, the Blackburn extracts were also tested with the Ames test to validate the new method alongside an existing process (Section 5.2). Mutagenicity data for the Blackburn extracts is discussed here followed by the C18/Silica/Isolute PAH HC extract data which includes a summary of all mutagenicity data for comparison purposes (Table 7.2).

7.4.1 Ames Testing of Blackburn Extracts with 80% S-9

The Blackburn extracts were tested for mutagenicity with 80% v/v S-9 (Section 5.2). The Blackburn and Grimmer methods were extracted using similar volumes of solvents. Mutagenicity was observed for all oils extracted by the Blackburn method at above 50 mg plate^{-1} without S-9 (as with the Grimmer extracts). There was an

increase however, of approximately 40% in the number of revertants observed on the addition of 80% v/v S-9 for oil 8 and Nytro-10GBN at 500 mg plate^{-1}. The Grimmer extracts had shown a greater increase in revertant number for oil 8 than Nytro-10GBN, which correlated with IP 346 % w/w data. Nevertheless the trend correlated sufficiently to indicate that both Grimmer and Blackburn extracts contained direct and indirect mutagens, as although indirect mutagens were only observed for one dose, they were present with both extracts. As many compounds are indirect mutagens it was not possible to conclude that the indirect mutagen was a PAH without analytical data, which was not available for the Grimmer and Blackburn extracts due to the complexity of the GC-MS chromatograms.

7.4.2 Ames testing of C18/Silica/Isolute PAH HC Extracts with 80% S-9

As the C18/Silica/Isolute PAH HC extraction method had been developed on a small scale, mutagenicity testing initially involved the use of a smaller version of the Ames test. The Miniscreen assay described by Brooks (1995) was such a version, but proved to be too small to allow conclusive growth of revertants on the plate (Section 5.3). When used with 80% v/v S-9 the plates became too opaque to read and were therefore inconclusive.

This lead to the scaling up of the C18/Silica/Isolute PAH HC for use with the Ames test. GC-MS analysis however, indicated that up scaling did not produce as clean an extract (Section 5.4). To determine if the discrepancies in extract efficiency would affect the mutagenicity of the oil extracts, the small scale extract and large scale extract were compared for mutagenicity. The small scale extract of Nytro-10GBN was extracted 20 times and the extracts combined to create the equivalent volume produced by the large scale method. The small scale extract of Nytro-10GBN showed the same level of indirect mutagenicity (~74-77 revertants) as the large scale extract (Section 5.5.3). This indicated that although the large scale extract contained more interferents, it was not affecting the mutagenicity measured with the Ames test and

the further improvement of the large scale extract was unnecessary for mutagenicity testing.

7.4.2.1 C18/Silica/Isolute PAH HC Comparison to Grimmer, 1P 346 and Blackburn Extracts

The mutagenicity results from the large scale extracts of the C18/Silica/Isolute PAH HC method showed a correlation with IP 346 % w/w data, and therefore the Grimmer and Blackburn extracts. All C18/Silica/Isolute PAH HC extracts were tested at 80% S-9 with the same *S. typhimurium* culture to ensure that reversion number could be directly compared. A particularly important observation was the absence of direct mutagenicity from the C18/Silica/Isolute PAH HC extracts. This indicated that the extraction had been successful at removing direct mutagens from the extract (Section 5.5.2). This reduced the background number of reversion observed on the plates and therefore improved the clarity with which an indirect mutagenic response was observed, ensuring that the indirect mutagenicity was not obscured by direct mutagenicity.

The C18/Silica/Isolute PAH HC extract of white oil showed no indirect mutagenicity whilst only a doubling in reversion number was observed for oil 4 and Nytro-10GBN at the highest dose tested. This doubling was found to be consistent at two doses (500 and 580 mg plate^{-1}) so both oils were considered mutagenic. Oil 4 had shown no indirect mutagenicity with Grimmer and Blackburn extracts so it was postulated that the presence of direct mutagenicity had previously obscured mild indirect mutagenicity. The oil 8 extract showed greater mutagenicity than the Nytro-10GBN extract. Unlike the Grimmer and Blackburn extracts, these extracts were analysed with GC-MS to determine the possible cause of the observed mutagenicity. As discussed in Section 7.6, PAHs were clearly the main component of the extract, and increases in PAH content (oil 8 > Nytro-10GBN > oil 4 > white oil) correlated with increases in reversion number, indicating that PAHs were the major cause of indirect mutagenicity.

On comparison of the Grimmer and Blackburn extracts with the C18/Silica/Isolute PAH HC extracts, the latter extracts produced a much smaller number of revertants (Table 7.2). Even if the Blackburn extracts for example, had not contained direct mutagens, oil 8 and Nytro-10GBN would still produce a reversion number of ~600 revertants plate^{-1}, whereas C18/Silica/Isolute PAH HC extracts only produced ~100 revertants. This was also true for the aged oils. The indirect mutagenicity observed with the C18/Silica/Isolute PAH HC aged extracts (~100 revertants) was not as great as that observed for Blackburn aged extracts (~400-600 revertants). This suggested that either the C18/Silica/Isolute PAH HC extraction was not extracting all mutagenic PAHs and therefore the complete mutagenicity was not observed, or the Blackburn extraction (and the Grimmer extracts) contained additional indirect mutagens which were not PAHs. As the C18/Silica/Isolute PAH HC extraction was shown to be efficient at extracting the large carcinogenic PAHs (Section 4.6) it was more probable that the Blackburn extract contained indirect mutagens other than PAHs. This cannot be proved due to the complex nature of the Blackburn extract, although the fact that the extract was so much more complex than the C18/Silica/Isolute PAH HC extract, added weight to the suggestion that it did contain additional indirect mutagens.

Alternatively, the direct mutagenicity could have enhanced the indirect mutagenicity of the Blackburn of Grimmer extract, therefore showing PAHs to be of greater threat. This indicates the importance of measuring PAH mutagenicity with the C18/Silica/Isolute PAH HC extraction with the absence of direct mutagenicity.

The findings of this investigation therefore indicate that the contribution of PAHs to oil mutagenicity was not as significant as suggested by the literature (Grimmer, 1983; McKee *et al*, 1989) for the particular oils tested in this work. A better indication of total oil mutagenicity was gained by measuring the total aromatic fraction. Therefore the aromatic content of these oils, extracted by LLE, which are known to contain components other than polyaromatics (Stang, 1993; 1999) may not be mainly subject to PAH mutagenicity, as sometime alluded in the literature (Grimmer, 1983; McKee *et al*, 1989; Järvholm and Easton, 1990; Brooks *et al.,* 1995). However, the PAHs that

contributed to oil mutagenicity, and that were potential environmental pollutants, had to be determined with GC-MS analysis as will be discussed in Section 7.6.

TABLE 7.2. Compiled results of the Ames data for all aromatic extracts. Mean revertant number for each oil extract is shown with (+) S-9 and without (-) S-9.

Oil	*Oil Extraction Methods*									
	IP 346 (10% S-9)		*Grimmer (10% S-9)*		*Grimmer (80% S-9)*		*Blackburn (80% S-9)*		*C18/Silica/ Isolute PAH HC (80% S-9)*	
	(-)S-9	*(+)S-9*	*(-)S-9*	*(+)S-9*	*(-)S-9*	*(+)S-9*	*(-)S-9*	*(+)S-9*	*(-)S-9*	*(+)S-9*
White	35	33	831	786	831	845	1274	1321	37	42
4	33	28	886	857	886	968	1221	1159	32	61
Nytro-10GBN	33	31	837	1037	837	1170	1017	1697	37	71
8	34	32	1228	1091	1228	2444	964	1569	36	99

7.4.3 Ames Testing of Aged Oil Blackburn and C18/Silica/Isolute PAH HC Extracts

The oil end-users expressed concerns about changes in oil mutagenicity with ageing, as PAH levels are believed to increase over the working life of an oil (Moret and Conte, 2000; Wang *et al.,* 2000; Wong and Wang, 2001). Oils were artificially aged (Section 2.1.1) for 1 week (ALT 1) 2 weeks (ALT 2) and 3 weeks (ALT 3). The aged oils were extracted with both the Blackburn and C18/Silica/Isolute PAH HC extraction along with the original oil (ALT 0).

The Blackburn aged oil extracts (Section 5.6.1) produced results that correlated with the Grimmer extracts (Section 3.5). All Blackburn extracts of ALT 0, ALT 1, ALT 2, and ALT 3 produced a similar direct mutagenic response indicating that oil composition did not alter enough with artificial ageing to affect mutagenicity

measured with the Ames test. In addition, revertant numbers increased from 550-850 revertants for oils on the addition of S-9, to 1000-1600 revertants, suggesting the presence of an indirect mutagen. Such direct and indirect mutagenicity was observed for used mineral oils by Granella *et al.,* (1995), although different oil were used, making a direct comparison of results inappropriate.

The C18/Silica/Isolute PAH HC extracts of ALT 0, ALT 1, ALT 2, and ALT 3 were also tested for mutagenicity and produced no direct mutagenesis. The C18/Silica/Isolute PAH HC method therefore, did not extract the additional direct mutagens found in Grimmer and Blackburn extracts. Due to the lack of background mutagenicity, the PAH results observed with S-9 was clearly mutagenic (~100 revertants). The mutagenicity however, did not alter sufficiently with artificial ageing to elicit change in mutagenic response from Ames test.

7.5 INHIBITION AND TOXIC EFFECTS FROM THE OIL AND OIL EXTRACTS

7.5.1 Inhibition

The oil and oil extracts were tested throughout the work to determine if the components of the oil affect PAH mutagenicity. The main concern, with respect to inhibition effects was that such compounds would disguise weakly mutagenic results.

The highest dose of each extract that did not produce a mutagenic response, with or without S-9 was added to the plate with benzo[a]pyrene and 2-amino anthracene so that only the mutagenicity of the benzo[a]pyrene or 2-amino anthracene would be observed. This was done to avoid the difficulty of subtracting benzo[a]pyrene/2-amino anthracene mutagenicity from oil extract mutagenicity.

The whole oil was tested (Section 3.3.3) and then the extracts from all the extraction methods examined were tested to determine if the extraction process increased or

decreased inhibition (Section 3.8 and 5.7). The inhibitions for oil and oil extracts were very similar; the greatest inhibition observed was ~32-40% for oil 8. As inhibition remained in the oil extract, it was concluded that the inhibitor must have been extracted with the PAHs. This inhibition effect was noted by Granella *et al.* (1995) with naphthenic mineral oils.

It was found that inhibition increased with aromatic content, suggesting that PAH content was the inhibiting factor. This explained why inhibition remained unaltered by extraction. Oil 8 reduced the revertant numbers of a mutagen by ~30%. White oil, which had no identifiable PAH content, resulted in a much lower reduction in revertant number. From analysing the composition of oil 8 by GC-MS (Section 6.3 and 6.4), it was observed that it did contain larger quantities of non-mutagenic PAHs than the other oils (Section 6.3.1 and 6.4.2). Non-mutagenic PAH inhibition of the enzymes was therefore postulated. Inhibition from mutagenic PAHs was unlikely as they may have their own mutagenic effect, thus increasing mutagenicity. However, Demarini (1998) noted that the mutagenicity of a PAH mixture will be dominated by the most mutagenic chemical, so no change in mutagenicity was more probable if other mutagens were competitively inhibiting benzo[a]pyrene.

Nordqvist *et al.*, (1981) showed that small, non-mutagenic PAHs such as phenanthrene were excellent substrates for S-9 enzymes. It was therefore possible that the non-mutagenic PAHs were being preferentially catalysed by the enzymes and hence interfering with mutagenicity. This did not correlate however, with work done with mineral oils by Hermann (1981) where mutagens were found to decrease the mutagenicity of benzo[a]pyrene and non-mutagens were found to enhance the mutagenicity even in an oily matrix solubilised by Tween 80, although the mechanism of enhancement is not clear. If Hermann (1981) is correct, the inhibition observed here must have been due to other factors that were not prevalent in the Tween 80/oil.

7.5.1.1 Inhibition at 80% S-9

To further determine if non-mutagenic PAHs were the source of inhibition, the S-9 concentration was increased to 80% v/v (Section 5.7.1). If non-mutagenic PAHs were interfering with enzyme active sites, increased activation enzyme concentration would increase the probability of activating a mutagen and therefore reduce inhibition. A reduction in inhibition was observed at elevated levels of S-9 indicating enzyme inhibition. Results in Table 5.8 also indicated that inhibition effects did not interfere with the data gained from the Ames test with 80% v/v S-9 since the inhibition (although still significant according to the t-test) was reduced for oil 8 to 2.6% from 26%. This reduction at elevated levels of S-9 was not observed by Granella *et al.,* (1995) who used naphthenic mineral oils common to the rubber industry. However, work done by Taylor *et al.* (1995) showed that S-9 concentration does have an affect on mutagenicity in a mixture. Furthermore, Hermann (1981) showed that the presence of PAHs decreases the indirect mutagenicity of benzo[a]pyrene but not the direct mutagen benzo[a]pyrene-4,5-oxide. This further implies that the cause of the observed reduction in revertants in the presence of 2-amino anthracene was inhibition of S-9 enzyme activation.

7.5.2 Toxicity

As inhibition affected the mutagenicity of the oil, it was possible that toxicity effects would also alter the mutagenicity observed. Toxicity would reduce the number of viable *S. typhimurium* cells that could be mutated and therefore lead to a reduction in revertant colonies and a false negative result in the Ames test. When testing a mixture of the EPA 16 priority PAHs for mutagenicity (Section 3.2.1.2) it was found that antagonistic effects were present and toxicity issues possibly disguised or cancelled out mutagenicity by reducing revertant numbers. This would lead to misinterpretation of positive results for negative results and illustrated the requirement for toxicity testing in conjunction with mutagenicity testing. As toxicity often causes extensive DNA damage, it was possible that mutagenicity would be most likely at doses lower

than the toxic dose. However, if the toxicity was due to factors unrelated to a mutagenic effect (Haroun and Ames, 1981), it was possible that the toxic effects would affect the mutagenic response observed.

For this reason the toxicity of all extracts was considered in relation to the corresponding Ames test data (Section 5.8). Rather than using a standardised toxicity test, *S typhimurium* was used to develop a toxicity test that would reflect toxicity effects with direct relevance to the Ames test.

The *S. typhimurium* culture was grown in nutrient broth and decreases in absorbance on oil extract addition were measured at 600 nm to determine toxic effects. No conclusive toxic effects were observed at the equivalent concentration of sample used in the Ames test for any of the oil extracts, so it was improbable that toxicity would have had an affect on the mutagenicity data gained. However, possible toxicity effects were observed at the highest two doses of all oils except white oil, suggesting there were toxic issues when using large quantities of oil. Further work is required to assess this potential hazard. In addition, as these toxic effects were observed at higher doses than those producing the mutagenic effect, the mechanism of toxicity is more likely to be due to acute DNA damage. There was a suggestion that PAH toxicity increases with alkylation (Irwin, 1997) and as the majority of PAHs in oil were later found to be methylated (Section 6.4), further investigations into the toxic threat to human health should be conducted.

With inhibition and toxicity issues with the Ames test resolved, the C18/Silica/Isolute PAH HC extracts were considered suitably characterised for PAH composition analysis by GC-MS.

7.6 TRENDS IN PAH CONTENT OF OILS 1-12

In order to assess the C18/Silica/Isolute PAH HC extraction's use in analysing the PAH content of oils, the data was compared to the IP 346 % w/w data provided by the National Grid Company Plc (Table 2.1) and immunoassay data. Immunoassay had previously been used as a PAH detection method at Cranfield University by Kim *et al.* (2001) and was shown to be a useful characterisation tool. Separate immunoassay kits were available for determining carcinogenic PAH content in addition to total PAH content, so immunoassay data also allowed the relationship between the total and carcinogenic PAH content of oils to be established.

7.6.1 Immunoassay

Immunoassay data for oils 1 to 12 was measured to compare with IP 346 % w/w data and the C18/Silica/Isolute PAH HC data. The results from kits specific to carcinogenic PAH were compared to kits measuring total PAH (Section 6.2.3). It was found that carcinogenic PAH increase correlated with total PAH increase, although it was evident that two oils with the same total PAH content would not necessarily have a similar carcinogenic PAH content. This showed that measuring total PAH with the IP 346 % w/w method may correlate with carcinogenicity, and indicated that measuring total PAH alone may eventually remove the requirement for mutagenicity testing for rapid screening purposes.

7.6.2 Comparing IP 346, Immunoassay and C18/Silica/Isolute PAH HC Extract Data

As the C18/Silica/Isolute PAH HC extracts were compatible with GC-MS analysis, this method was used to identify and quantify PAHs in transformer oil. The total peak area of the TIC and SICs were integrated and analysed as to whether the C18/Silica/Isolute PAH HC extraction method could be used as a replacement for

both the IP 346 measure of total aromatics, or the immunoassay measure of total PAH content (Section 6.3.1). The replacement of either method is desirable, as IP 346 is laborious to perform, and the immunoassay approach is expensive for the end-user if routinely used.

A correlation between TIC integration and IP 346 data was observed. A correlation was also evident for immunoassay and IP 346, although there was a slight variation in the trend for oils 3 and 4. The SIC data did not correlate as well as the TIC data for the immunoassay and IP 346 methods. Oil 2 and 3 had a lower PAH content than oil 1 by SIC whilst the TIC, IP 346 and immunoassay data all indicated that oil 1 had the lowest PAH content. The total integration of TIC area was therefore a significant indicator of total PAH content and correlated more closely with the IP 346 % w/w industrial standard method and the immunoassay approach. In addition, as the C18/Silica/Isolute PAH HC TIC integration method yielded a correlation with both IP 346 % w/w and immunoassay methods, it was clear that total PAH content (measured by immunoassay) and total aromatic content (measured by IP 346) were linked. This confirmed that the IP 346 % w/w method, although a crude extraction method, did give a valid indication of both total aromatics and total PAH content and along with its correlation with skin painting tests (Stang, 1993; 1999), explains why it is an industry standard. However, IP 346 could not identify or quantify specific PAHs (or other mutagens) in the oil and in this respect the C18/Silica/Isolute PAH HC extraction may be considered an improvement over the IP 346 % w/w method.

7.6.3 Identification of PAHs in Oil

The TICs of the C18/Silica/Isolute PAH HC extracts were also used to identify the PAHs naturally present in the oil (Section 6.4). The mass spectrometric data was used to determine the most compatible structure for those compounds found. In this case the NIST MS library (part of the GC-MS Turbomass software) was used for comparison. The majority of PAHs present were methylated fluorenes and phenanthrenes, eluted at retention times between naphthalene and chrysene and

agreed with the work of Wang *et al.,* (2000). The exact isomers present could not be determined by GC-MS without comparison with standards. However, such an approach is not feasible given that there may be hundreds of naturally occurring PAH isomers found in transformer oil.

Interestingly, the heterocyclic dibenzothiophene was also found in the extracts and was considered as a PAH in this study due to its abundance and concerns over its potential toxicity and mutagenicity. Other PAHs identified that may also contribute to mutagenicity were methyl chrysene, benzo[g,h,i]fluoranthene and dimethyl-benzo[c]phenanthrene, although the mutagenicity of the latter two compounds has not been conclusively established (IARC, 2002).

The EPA 16 priority PAHs were also identified (Section 6.4) with TIC and quantified (Section 6.5) using SIC to reveal PAHs obscured in the TIC by overlapping peaks. Fluorene, phenanthrene, anthracene, pyrene and benzo[a]anthracene were the main EPA 16 priority PAHs identified, although absolute values varied greatly between oils. Benzo[k]fluoranthene was also identified although the amounts found were below the detection limits of the calibration profile.

From this analysis it was concluded that the probable causes of indirect mutagenicity were benzo[a]anthracene, dibenzothiophene (and its methylated forms), benzo[g,h,i]fluoranthene, methyl chrysene, dimethyl-benzo[c]phenanthrene and possibly benzo[k]fluoranthene. Many of the methylated PAHs found in the oil have previously been assumed to present the same hazards as unmethylated PAHs (Irwin, 1997). However, this assumption is questionable since 5-methyl chrysene, for example shows an enhancement of mutagenicity due the methyl group being present in the bay region (Harvey, 1991). Thus, should the methyl chrysene found in the oil be 5-methyl chrysene, it would certainly contribute towards mutagenicity. This highlights the requirement for thorough determination of individual isomers and the consequences of incorrect identification. It cannot be concluded therefore that the methylated PAHs do not contribute towards mutagenicity, especially in such complex mixtures.

7.6.4 Identification and Quantification of PAHs in Aged Oil

By analysing the C18/Silica/Isolute PAH HC extracts by GC-MS, identification and quantification of PAHs was monitored for changes with age. This gave an indication of whether the PAH composition of the oil changed while in service and whether ageing led to a further build up of mutagenic PAHs, which would increase the risk to the end-user. As the oils had been artificially aged prior to acquisition, they could not be spiked before ageing and those EPA 16 priority PAHs naturally occurring in the oils were the only PAHs selected for quantification.

Initial assessment of total PAH content was performed by integrating the total area of the TIC. ALT 0 oil contained the most PAHs according to this measurement and PAH contents showed a general decreased with artificial ageing of up to 3 weeks (Section 6.6). If this trend in total PAH was correct, ALT 0 oil would also contain the greatest amount of carcinogenic PAHs according to the trend seen for immunoassay data (Section 6.2.3).

According to the quantitative data, phenanthrene, fluoranthene and pyrene, were all present in the oil and showed a general increase in quantity with ageing. Pyrene and phenanthrene increased after 3 weeks of ageing to twice their original amount while fluoranthene increased by three times (Section 6.6.2). The literature suggests that organic compounds partially break down at high temperatures into smaller unstable fragments that can recombine into PAHs and that two and three ringed PAHs accumulate particularly quickly in used lubricating oils (Wang *et al.,* 2000; Wong and Wang, 2001). Aromatisation at lower temperature can also occur, given sufficient time to create alkylated PAHs (Moret and Conte, 2000). The PAHs themselves are very stable and so are less prone to breakdown, making it more likely that PAH content should increase with age than decrease. This explained the increase observed for phenanthrene, pyrene and fluoranthene. However, as the opposite trend had been observed when total PAH content was monitored by TIC integration (Section 6.6.1),

the TIC was not a good indicator of PAH contents for aged oils. Nevertheless, the TIC integration method was shown to be effective for new oils 1 to 12 (Section 6.3.1) and it was postulated that the reduction in TIC area of aged oils was due to the more complex oil matrices overloading the extraction sorbents (Wang *et al.*, 2000). Thus, less oil sample should be used in the extraction of aged oils in future.

7.6.5 Measuring the PAH Content of Non-transformer Oils using the C18/Silica/Isolute PAH HC Extraction

The C18/Silica/Isolute PAH HC method was used to extract PAHs from a range of mineral and edible oils (Section 6.7) to ascertain if this method could be used to determine the PAHs in a wide range of oil matrices. Red diesel and petrol were tested as they were known to have a high PAH content. Edible oils such as Olive oil were tested for their low PAH content, observed by Márquez-Ruiz *et al.* (1996). However, due to their lipophilic nature, edible oils are often contaminated with PAHs from plant exposure to vehicle exhaust emissions, originating from fuels such as diesel and petrol, industrial emissions or the packing process (Moret and Conte, 2000).

The red diesel and petrol extracts were found to contain a mixture of PAHs, suggesting that the method can be extended to relatively assess the PAH content of general oils. The edible oils, as expected, contained no quantifiable PAHs, suggesting that the refining processes used to produce the oil were sufficient to remove contaminating PAHs (Moret and Conte, 2000).

7.7 OVERALL CONCLUSIONS OF THE THESIS

The main findings of this work are briefly outlined and suggestion are made as to how these results affect transformer oil end-users and how resultant concerns may be addressed.

- A variety of PAHs including methylated fluorenes and phenanthrenes were identified in transformer oil (Table 6.7). The mutagenicity and toxicity of these PAHs is not well understood due to the large number of alkylated forms. Therefore it cannot be assumed that theses alkylated forms are only as hazardous as the non-alkylated parent PAH (Irwin, 1997). The testing of all the PAHs identified in this study would be a very significant undertaking, thus all sources of mutagenicity could not be individually assessed. Nevertheless, all PAHs are prevalent in the environment, so all the PAHs in oil are of concern if they should leak from transformers. Accumulation of the carcinogenic PAHs in particular would cause the greatest impact to the general environment and human health. For this reason, the transformer oils with lower PAH content (oils 1, 4, 7, 11 and Nytro-10GBN) should be preferentially employed for their lower overall PAH content and lowest carcinogenic PAH content.

- PAHs were found to contribute to the total mutagenicity observed in the oil but were not found to be the main source. The mutagenicity observed with the Grimmer (Section 3.4.2) and Blackburn (Section 5.2) extracts was mostly caused by direct mutagens and therefore were not attributable to PAHs. Of those indirect mutagens measured, the reversion number was significantly reduced in the presence of the PAH specific C18/Silica/Isolute PAH HC extracts (Section 5.5.2) when compared to the Grimmer or Blackburn extracts. This suggested that the additional indirect mutagenicity observed for the Grimmer and Blackburn extracts was due to compounds other than PAHs. As PAHs were not the main cause of mutagenicity in the oil in this case, removing them from the oil would not reduce the hazard associated with oil handling but would reduce the in-service lifetime of

the oil as the PAHs reduce oil deterioration. For this reason, removal of PAHs from the oil is immaterial. Nevertheless, using transformer oils with the lowest mutagenic PAH content (oil 1, 4, 7, 11 and Nytro-10GBN) would be a preferred option.

- Dibenzothiophene, methylated dibenzothiophene, benzo[g,h,i]fluoranthene, dimethyl-benzo[c]phenanthrene, benzo[a]anthracene and possibly benzo[k]fluoranthene (found at low concentrations) were identified in the oil and were considered to contribute to the mutagenicity of transformer oil. Oils 1, 4, 7, 11 and Nytro-10GBN, as well as having the lowest overall PAH content, were not found to contain any of these compounds (Table 6.7) and thus may prove the most suitable oils to use in order to reduce PAH-specific carcinogenic risk factors. Using these oils will not remove the threat imposed from the direct mutagens however, as these were present in all oils tested (including white oil) with the Ames test.

- The presence of non-mutagenic PAHs was found to be the probable cause of PAH-induced mutagenic inhibition (discussed in Section 7.5). The revertant number produced in the presence of a strong mutagen decreased when an oil extract with high non-mutagenic PAH content was added. The higher the level of PAH in the oil extract, the more inhibition was observed. It was postulated that non-mutagenic PAHs were preferentially interacting with the active site of the enzyme, thereby inducing some form of inhibition. Increasing the levels of S-9 from 10% to 80% v/v confirmed this suggestion (Section 5.7.1). The presence of inhibiting non-mutagens would therefore suggest a reduction in the impact of the mutagenic threat to humans when handling the oil. However, the process is complicated in that mammalian skin and liver cells contain an excess of these activating enzymes, so inhibition would be minimised and offer no protection from mutagenesis.

- Total PAH content measured by immunoassay was found to relate to carcinogenic PAH content (Section 6.2.3). This indicated that the IP 346 % w/w method of

determining total PAH may well correlate with carcinogenicity, so that total PAH may be used to predict the risk of carcinogenicity from the oil. However, it was found that although an oil with higher total PAH content would in all probability contain a higher carcinogenic content, two oils that contained the same total PAH content would not necessarily exhibit the same carcinogenic PAH content.

- The new C18/Silica/Isolute PAH HC extraction offered benefits over those PAH extraction methods reported in the literature. The method was found to be effective at determining the mutagenicity of PAHs in oil when used with the Ames test (Section 5.5.2). The extracts were sufficiently clean for individual PAH determination by GC-MS, unlike the Blackburn, Grimmer and IP 346 methods and also by definition, the total and carcinogenic immunoassay methods. Only the C18/Silica/Isolute PAH HC extracts were conclusive for PAH identification (Section 6.4) or quantification (Section 6.5) due to a reduction in interference in the sample. A further application exclusive to the GC-MS analysis of the C18/Silica/Isolute PAH HC extracts was the determination of total PAH content (Section 6.3.1) and provided a simple, rapid, less hazardous and less expensive method that directly fulfils the criteria of IP 346 % w/w estimation.

- Possible toxic effects were observed in oils 8, 4 and Nytro-10GBN, although they did not affect the Ames test at the doses of extract tested (Section 5.8). This suggested that had the oil extracts been tested to a higher dose, toxicity effects would have been observed. Toxic effects are of as much concern as mutagenicity for users, especially as the oil is used in large quantities and so must be investigated further.

- Ageing of the oil was found to affect the PAH composition (Section 6.6). The amount of individual PAHs increased by between 2 and 3-fold following 3 weeks of ageing. Mutagenicity was not affected by ageing however, according to the Ames test (Section 3.5 and 5.6). This was possibly because the observed increase in PAHs levels were due to the smaller non-mutagenic PAHs as noted by Wang *et al.* (2000) and Wong and Wang (2001).

7.8 FURTHER WORK

A number of interesting points emerge from the discussion above which merit further investigation. Such further work is outlined here.

- The C18/Silica/Isolute PAH HC extracts should be concentrated further (by extracting more oil into a small volume of solvent) and analysed with GC-MS. PAHs that were present in very small quantities, and therefore not identified by previous studies, could be identified and quantified.

- As many of the PAHs in the oil extracts have been identified using the NIST library with GC-MS, HPLC may be used to separate and identify the different isomers present, as some may present a greater mutagenic threat than others.

- Standards of those PAHs identified in the oil could be tested for mutagenicity to determine which cause the mutagenicity in oil. It was been suggested that dibenzothiophene methylated benzothiophene, benzo[g,h,i]fluoranthene, dimethyl-benzo[c]phenanthrene, benzo[a]anthracene and possibly benzo[k]fluoranthene may contribute to mutagenicity, but testing standards would conclusively confirm if this was the case. Methylated fluorene and phenanthrene would also require mutagenicity testing once the isomers were resolved, to determine their individual contributions.

- The effects of complex mixtures of PAHs on mutagenicity should be explored. Although the oil extract from the C18/Silica/Isolute PAH HC method were mainly composed of PAHs, there was still a possibility that indirect mutagens were also present. Ideally, as PAH quantification of the oil may now be possible, it would be useful to recreate the PAH mixture in the oil, using standards in the correct quantities, and test the mixtures for mutagenicity. It would be interesting to observe if the resultant mutagenicity correlated with that seen in the oil extract and would also support the use of the use of the C18/Silica/Isolute PAH HC extracts for PAH mutagenicity testing.

- Improvement in the application of the C18/Silica/Isolute PAH HC extraction method would benefit the oil users through the identification and quantification of PAHs in oil. The method would benefit from system automation, which would increase the speed of the extraction method and would improve the repeatability of the solid phase extraction method through the application of a constant displacement pressure. The extraction system could be directly attached to the GC-MS for high samples throughput and reduce the need for highly trained staff. The system may also be miniaturised using solid phase sorbent disks (Thurman and Mills, 1998). Ultimately, a field-based system incorporating a portable GC-MS system may be envisaged.

- Use of an alternative, preferably small scale, mutagenicity assay with the small scale C18/Silica/Isolute PAH HC extraction, would provide further useful data on transformer oil mutagenicity. An example is the yeast mutagenicity test (Gentronix, 2001) which is automated and conducted in microtitre plates. The yeast assay has the benefit of employing an eukaryotic system, which related more closely to the mutagenicity observed in humans.

- Developing the large scale C18/Silica/Isolute PAH HC further, for use with the Ames test or other mutagenicity or toxicity tests may be useful in situations where a small scale mutagenicity tests is not appropriate. Although the inefficiencies observed when using the large scale extraction did not effect the mutagenicity results, this extraction may be developed further to improve extraction efficiency. This would preferably involve the use of larger Isolute PAH HC columns, and would require co-operation from the sorbent supplier.

- Further toxicity testing, with a standardised toxicity test kit, would determine if there was any acute hazards involved in using transformer oil, which was suggested to be present from the toxicity observed at high doses of oil with *S. typhimurium*.

- Finally, the affects of ageing on PAH composition should be investigated further. PAHs could be aged individually to see the effects of mutagenicity, in addition to the ageing of a mixture. Ideally, a further study is required with oil that was spiked with all 16 EPA PAHs at representative concentrations before ageing, to obtain more conclusive results. It is possible that individual PAHs will be affected by the ageing process to differing extents.

REFERENCES

Alberts, B; Bray, D; Lewis, J; Raff, M; Roberts, K; Watson, J.D. (1989). Molecular biology of the cell. 2nd ed. Garland Publishing Inc.

Ames, B.N; Mccan, J; Yamasaki, E (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. *Mutation Research*, **31**, 347-364.

Andrews, A.W; Thibault, L.H; Lijinsky, W. (1978). The relationship between carcinogenicity and mutagenicity of some polynuclear hydrocarbons. *Mutat. Res.* **51**, 311.

Barceló, D; Oubiña, A; Salau, J.S; Perez, S. (1998). Determination of PAHs in river water samples by ELISA. *Analytica Chimica Acta,* **376**, 49-53.

Bekaert, C; Rast, C; Ferrier, V; Bispo, A; Jourdain, M.J; Vasseur, P. (1999). Use of the in vitro (Ames and Mutatox tests) and the in vivo (amphibian micronucleus tests) assays to assess the genotoxicity of leachates from a contaminated soil. *Organic geochemistry,* **30,** 953-962.

Bingham, E (1988). Carcinogenicity of mineral oils. *Annals of the New York academy of Sciences*. **534,** 452-458.

Blackburn, G.R; Deitch, R.A; Schreiner, C.A; Mehlman, M.A; Mackerer, C.R. (1984). Estimation of the dermal carcinogenic activity of petroleum fractions using a modified Ames assay. *Cell boil. Toxicol.,* **1**, 67-80.

Blackburn, G.R; Deitch, R.A; Schreiner, C.A; Mehlman, M.A; Mackerer, C.R. (1986). Predicting carcinogenicity of petroleum distillation fractions using a modified *salmonella* mutagenicity assay. *Cell biol. Toxicol.,* **2 (1)**, 63-84.

Boffetta, P (1993). Electricity production and cancer risk. *The Cancer Journal*, **6 (1),** *Citing Internet resources* (www document).

http://www.infobiogen.fr/agora/journals/cancer/articles/6-1/boff.htm (accessed 12 November 1999)

Brook, T.M (1995). The use of a streamlined bacterial mutagenicity assay, the MINISCREEN. *Mutagenesis*, **10 (5),** 447-448

Brooks, T.M; Priston, R.A.J; Wright, A.S; Watson, W.P (1995). Evaluation of modified bacterial mutagenicity assays for the genotoxicity testing of mineral oils. *Mutagenesis*, **10 (5),** 409-415.

BS 2000 Part 346 (1996). Determination of polycyclic aromatics in unused lubricating base oils and asphaltene free petroleum fractions- dimethyl sulphoxide extraction refrative index method. *The British Standard Institute*, London, UK

BS EN 61125 (1993). Unused hydrocarbon based insulating liquids- test methods for evaluating the oxidation stability. *The British Standard Institute*, London, UK

Buening, M.K; Levin, W; Karle, J.M; Yagi, H; Jerina, D.H; Conney, A.H (1979). Tumorigenicity of bay region epoxides and other derivatives of chrysene and phenanthrene in newborn mice. *Cancer Research,* **39**, 5063-5068.

Burke, D.A; Wedd, D.J; Burlinson, B (1996). Use of the Miniscreen assay to screen novel compounds for bacterial mutagenicity in the Pharmaceutical industry. *Mutagenesis*, **11 (2),** 201-205

Chuang, J.C; Pollard, M.A; Chou, Y-L; Menton, R.G; Wilson, N.K. (1998*). The Science of the Total Environment*, **224,** 189-199.

Coombs, M; Dixon, C; Kissonerghis, A.M. (1976). Evaluation of the mutagenicity of compounds of known carcinogenicity, belonging to the benz[a]anthracene, chrysene, and cyclopenta[a]phenantrene series, using Ames test. *Cancer res.* **36**, 4525.

De Flora, S; Zanacchi, P; Camoirano, A; Bennicelli, C; Badolati, G.S (1984). Genotoxic activity and potency of 135 compounds in the Ames reversion test and in bacterial DNA-repair test. *Mutation Research*, **133**, 161-198.

DeMarini, D. (1998). Mutation spectra of complex mixtures. *Mutation research*, **411**, 11-18.

Environmental Protection Agency (EPA) USA (2002). *Citing Internet resources* (www document)
http://www.epa.nsw.gov.au/air/dopahhm/pahintroduction.htm
(accessed 11 December 2002)

Fessenden, R.J. & Fessenden, J.S. (1994). Organic Chemistry. Fifth ed. Brooks/Cole.

Gentronix website (2001). *Citing Internet resources* (www document).
http://www.gentronix.co.uk (accessed 17 December 2001)

Glucker, J.P; Carrell, H.L; Katx, A.Afshar, C.E (1999). Structual studies of PAHs and their metabolites. *Polycyclic aromatic compounds,* **14**, 87-98.

Gollapudi, B; Krishna, G (2000). Practical aspects of mutagenicity testing strategy: and industrial perspective. *Mutation Research*, **455,** 21-28.

Granella, M; Clonfero, E (1991). The mutagenic activity and polycyclic aromatic hydrocarbon content of mineral oils. *Int. Arch. Occup. Environ. Health*, **63**, 149-153.

Granella, M; Ballarin, C; Nardini, B; Marchioro, M; Clonfero, E (1995). Mutagenicity and contents of polycyclic aromatic hydrocarbons in new high-viscosity naphthenic oils and used and recycled mineral oils. *Mutation Research*, **343**, 145-150.

Grimmer, G; Jacob, J; Naujack, K-W (1981). Profile of the polycyclic aromatic hydrocarbons from lubricating oils inventory by GCGC/MS- PAH in environmental materials, part 1. *Fresenius Z. Anal. Chem*. **306**, 347-355

Grimmer, G; Dettbarn, G; Brune, H; Deutsch-Wensel, R; Misfield, J. (1982). Quantification of the carcinogenic effect of polycyclic aromatic hydrocarbons in used engine oil by topical application onto the skin of mice. *Int. Arch, occup. Environ. Health*, **50,** 95-100.

Grimmer, G (ed) (1983). Environmental carcinogens: polycyclic aromatic hydrocarbons. CRC Press.

Grimmer, G; Brune, H; Dettbarn, G; Jacob, J; Misfeld, J; Mohr, U; Naujack, K-W; Timm, J (1991). Relevance of polycyclic aromatic hydrocarbons as enviromental carcinogens. *Fresenius Journal of Analytical Chemistry*, **339,** 792-795.

Haroun, L; Ames, B.N (1981). The Salmonella mutagenicity test: an overview. From *Short Term Tests for Chemical Carcinogens*, Stich, H.F (ed), Springer-Verlag

Harvey, R.G. (1991). Polycyclic Aromatic Hydrocarbons Chemistry and Carcinogenicity. Cambridge University Press.

Henry, M (2000). SPE technology-principles and practical consequences. From Solid phase extraction: principles, techniques and applications Simpson, N.J.K (ed), Marcel Dekker

Hermann, M; Chaude, O; Weill, N; Bedouelle, H; Hofnung, M (1980). Adaptation of the Salonella/mammalian microsome test to the determination of the mutagenic properties of mineral oils. *Mutation research*, **77**, 327-33

Hermann, M. (1981). Synergistic effects of individual polycyclic aromatic hydrocarbons on the mutagenicity of their mixtures. *Mutation Research*, **90,** 399-409.

Hewstone R.K. (1994). Health, safety and environmental aspects of used crankcase lubricating oils. *The science of the total environment*, **156,** 255-268.

Ingram, A.J; Scammells, D.V; May, K (1994). An investigation of the main mutagenic components of a carcinogenic oil by fractionation and testing in the modified Ames assay. *Journal of applied toxicology*, **14 (3),** 173-179.

International Agency for Research on Cancer (IARC) (2002) Monograph website. Polynuclear aromatic hydrocarbons Part 1 and 2. **32-33**, *Citing Internet resources* (www document). http://193.51.164.11/monoeval/allmonos.html (accessed 14 March 2003)

Irwin, R.J; (1997). Environmental contaminants encyclopedia, dibenzothiophene entry. National Park Services. *Citing Internet resources* (www document). http:/www.nature.nps.gov/toxic/dibenzot.pdf (accessed 25 January 2003)

ISO 10712 (1995). Water quality- *Pseudomonas putida* growth inhibition test (*Pseudomonas* cell multiplication inhibition test). *International organisation for Standardisation.*

Jackson, B.A; Pertel, R (1986) "Regulatory implications of Ames' mutagenicity assay using *Salmonella typhimurium*," *Fundemental and applied toxicology*, **7,** 1-16

Jansson, L.I (1992) Rising demands on mineral oil: oil harmless when handled properly. *Naphthenics magazine,* **1**. *Citing Internet resources* (www document). http://www.marathon.se/nynas/start/article.cfm?art_ID=767 (accessed 25 July 2001)

Järvholm, B. Easton, D. (1990). Models for skin tumour risks in workers exposed to mineral oils. *British Journal of Cancer*, **62**, 1039-1041.

Johnson, B.T (1992). An evaluation of a genotoxicity assay with liver S-9 for activation and luminescent bacteria for detection. *Environmental Toxicology and Chemistry,* **11,** 473-480.

Josephy, P.D; Gruz, P; Nohmi, T. (1997). Recent advances in the construction of bacterial genotoxicity assays. *Mutation Research*, **386**, 1-23.

Kim, I.S,. Setford, S.J, Ritchie, L.J, Taylor, J, Allen, M and Saini, S (2001). Quantitative immunoassay for polynuclear aromatic hydrocarbons in electrical insulating oils. *Analytica Chimica Acta* **21587**, 1-13.

Kitson, F.G; Larsen, B.S; McEwen, C.N. (1996) Gas chromatography and mass spectroscopy. A practical guide. Academic Press.

Kroon, I (1989). Nynas uses in-house method to analsye PAC content. *Naphthenics magazine Citing Internet resources* (www document). http://www.marathon.se/nynas/start/article.cfm?Art_ID=759 (accessed 25 July 2001)

Lindroth, A (1995). The transformer- a tried and tested product, with scope for development. Naphthenics, **4**, Nynas. *Citing Internet resources* (www document). http://www.marathon.se/nynas/start/article.cfm?Art_ID=774 (accessed 25 July 2001)

Lodovici, M; Akpan, V; Giovannini, L; Migliani, F; Dolara, P. (1998). Benzo[a]pyrene diol-epoxide DNA adducts and levels of polycyclic aromatic

hydrocarbons in autoptic samples from human lungs. *Chemico-Biological interactions,* **116,** 199-212.

Manahan, S.E. (1994). Environmental chemistry, 6th edition, CRC Press, Inc.

Marcé, R.M; Borrull, F (2000) Solid-phase extraction of polycyclic aromatic hydrocarbons. *Journal of chromatography A*, **885**, 273-290.

Maron, D.M; Ames, B.N (1983). Revised methods for the *Salmonella* mutagenicity test. *Mutation Research,* **113**, 173-215.

Márquez-Ruiz, G; Jorge, N; Martin-Polvillo, M; Dobarganes, M.C. (1996). Rapid, quantitative determination of polar compounds in fats and oils by solid-phase extraction and size-exclusion chromatography using monostearin as internal standard. *Journal of Chromatography A*, **749**, 55-60.

McCann, J; Choi, E; Yamasaki, E; Ames, B.N. (1975). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals, Proceedings of the National Academy of Sciences USA, **72**, 5135-5139.

McKee, R.H; Daughtrey, W.C; Freeman, J.J; Frederici, T.M; Phillips, R.D; Plutnick, R.t (1989). The dermal carcinogenic potential of unrefined and hydrotreated lubrication oils. *Journal of toxicology*, **9 (4),** 265-270.

McNair, H.M; Miller, J.N. (1998). Basic Gas chromatography. Wiley and Sons, Inc.

Mendelsohn, M.L (1988). Can chemical carcinogenicity be predicted by short-term tests? *Annals of the New York Academy of Sciences*, **534,** 115-126

Miller, J.N; Miller, J.C (2000). Statistics and chemometrics for analytical chemistry. 4th Edition, Prentice Hall (Pearson Education Limited).

Moret, S; Conte, S (2000). Polycylic aromatic hydrocarbons in edible fats ans oils: occurance and analytical methods. *Journal of Chromatography A*, **882**, 245-253

National Grid Company Plc. website (2002). *Citing Internet resources* (www document) http://www.nationalgrid.co./uk/ (accessed July 25 2002)

Niessen, W.M.A (2001) Current practice of gas chromatography-mass spectroscopy. Vol 86, Chromatographic Science Series, Marcel Dekker, New York.

Noordkamp, E.R; Grotenhuis, J.T.C; Rulkens, W.H (1997). Selection of an efficient extraction method for the determination of polycyclic aromatic hydrocarbons in contaminated soil and sediment. *Chemosphere*, **35 (9),** 1907-1917.

Norqvist, M; Thakker, D.R; Vyas, K.P; Yagi, H; Levin, W; Ryan, D.E; Thomas, P.E; Conney, A.H; Jerina, D.M (1981). Metabolism of chrysene and phenanthrene to bay-region diol expoxides by rat liver enzymes. *Molecular Pharmacology,* **19**, 168-178

Nynas (1999). Health and safety aspects of naphthenic oil. *Citing Internet resources* (www document). http://www.nynas.com/naphthenics (accessed 24 May 2000)

Nynas, (2001). Transformer oil Handbook. *Citing Internet resources* (www document). http://www.nynas.com/naphthenics (accessed 24 May 2000)

OECD guidelines for testing of chemicals (1983). Genetic toxicology: *Salmonella typhimurium* reverse mutation assay. **471**

Pahlavanpour, B; Wilson, G (1999). Fingerprinting of transformer oil by GC-MSD. *CIGRE 15.01.06* Study of techniques for fingerprinting oils, internal report, National Grid Company Plc.

Paschke, A; Herbel, W; Steinhart, H (1992). Determination of mono- to tetracyclic aromatic hyrocarbons in lubricating oil. *Journal of High Resolution Chromatography*, **15**, 827-833.

Phillips, D.H (1999). Polycyclic aromatic hydrocarbons in the diet. *Mutation Research*, **443,** 139-147

Polytechnic University, Department of Chemistry and Chemical Engineering USA website, gas chromatography. *Citing Internet resources* (www document). http://www.accessexcellence.org/TSN/SS/chromatography_background.html (accessed 12 February 2003)

Poole, C.F; Poole, S.K (2000). Theory meets practice. From Solid phase extraction: principles, techniques and applications Simpson, N.J.K (ed), Marcel Dekker

Purchase, I.F.H; Longstaff, E; Ashby, J; Styles, J.A; Anderson, D; Lefevre, P.A; Westwood, F.R. (1976). Evaluation of six short-term tests for detecting organic chemical carcinogens and recommendations for their use. *Nature,* **264,** 624.

Purchase, I.F.H; Longstaff, E; Ashby, J; Styles, J.A; Anderson, D; Lefevre, P.A; Westwood, F.R. (1978). Evaluation of six short term tests for detecting organic chemical carcinogens. *BR. J. Cancer,* **37**, 873.

Purdue University, West Lafayette, IN (2002) website, chromatography. *Citing Internet resources* (www document). http://chemed.chem.purdue.edu/analyticalreview/chromatography/index.html (accessed 12 February 2003)

Raineri, R; Poiley, J.A; Pienta, R.J; Andrews, A.W (1981). Metabolic activation of carcinogenic in the *Salmonella* mutagenicity assay by hamster and rat liver S-9 preparations. *Environmental Mutagenesis*, **3,** 71-84.

Randerath, K; Randerath, E; Zhou, G.D. Supunpong, N; He, L.Y; McDonald. T.J; Donnelly, K.C. (1999). Genotoxicity of complex mixtures recovered from contaminated lake sediments as assessed by three different methods. *Environmental and Molecular Mutagenesis*, **33**, 303-312.

Roy, T.A; Blackburn, G.R; Mackerer, C.R. (1996) Evaluation of physiochemical factors affecting dermal penetration and carcinogenicity potency of mineral oils containing polycyclic aromatic compounds. *Polycyclic Aromatic Compounds*, **10**, 333-342.

Salamone, M.F; Heddle, J.A; Katz, M (1979). The mutagenic activity of thirty polycyclic aromatic hydrocarbons (PAH) and oxides in urban airborne particulates. *Environment International*, **2**, 37-43.

Sargenti, S.R; McNair, H.M (1998) *Journal of Microcolum*, **125**. Cited in: Marcé, R.M; Borrull, F (2000) Solid-phase extraction of polycyclic aromatic hydrocarbons. *Journal of chromatography A*, 885, 273-290.

Schneider, K; Roller, M; Kallberlah, F; Schuhmacher-Wolz, U. (2002). Cancer risk assessment for oral exposure to PAH mixtures. *Journal of Applied Toxicology*, **22,** 73-83.

Shou, M; Korzekwa, K.R; Crespi, C.L; Gonzalez, F.J; Gelboin, H.V. (1994). The role of 12 cDNA-expressed human, rodent, and rabbit cytochromes P450 in the metabolism of benzo[a]pyrene and benzo[a]pyrene trans-7,8-dihydrodiol. *Mol. Carcinog.*, **10**, 159-168.

Stang, G (1993). Various methods of determining PCA content. *Naphthanics magazine*, **2**. *Citing Internet resources* (www document). http://www.marathon.se/nynas/start/article.cfm?Art_ID=768 (accessed 25 July 2001)

Stang, G. (1999) PAC varies with the method of measuring. *Naphthanics magazine: Special issue on transformer oil,* Nynas, Sweden.

Stang, G (2000). Naphthenic oils less carcinogenic. *Naphthenics Market Magazine*, **3**. *Citing Internet resources* (www document). http://www.marathon.se/nynas/start/article.cfm?Art_ID=151&Iss_ID=63 (accessed 25 July 2001)

Tauc, M; Herman, M; Dansette, P.M; Vandecasteele (1984). Enzymatic characterisation of the polynuclear aromatic hydrocarbons activating rat-liver preparations used in the mutagenicity test of Ames. *Mutation Research,* **125**, 123-133

Taylor, M.S; Setzer, R.W; DeMarini, D.M. (1995). *Mutation Research*, **335**, 1-14. Cited in: White, P. A. (2002). The genotoxicity of priority polycyclic aromatic hydrocarbons in complex mixtures. *Mutation Research*, 515, 85-98.

Thurman, E.M and Mills, M.S (1998). Solid-Phase Extraction, Principles and Practice. *Chemical Analysis, a series of monographs on analytical chemistry and its applications*, **147**, John Wiley and Sons Inc.

Tolbert, P.E. (1997). Oils and Cancer. *Cancer Causes and Control*, **8**, 386-405.

Toxciological Research Centre (TRC) (Hungary) website. *Citing Internet resources* (www document). http://www.trc.hu/envionmental/environmental.html (accessed 12 February 2003)

Wang, J; Jia, C.R; Wong, C.K; Wong, P.K (2000). Characterization of polycyclic aromatic hydrocarbons created in lubricating oils. *Water, Air and Soil Pollution,* **120**, 386-396.

Watson, W.P; Brooks, T.M; Gonzalez, L.P; Wright, A.S. (1985) "Genotoxicity studies with mineral oils; effects of oils on the microbial mutagenicity of precursor mutagens and genotoxic metabolites" *Mutation research*, **149**, 159-170.

Walmsley, R.M; Billinton, N; Heyer, W.D (1997). Green fluorescent protein as a repoter gene for the DNA damage-induced gene *RAD54* in *Saccharomyces*. *Yeast,* **13**, 1535-1545.

Weaver, R.F. and Hedrick, P.W. (1995). Basic Genetics. Second Edition, Wm C. Brown Publishers.

Wells, M.J.M. (2000). Handling large volume samples: applications of SPE to environmental matrices. From Solid phase extraction: principles, techniques and applications Simpson, N.J.K (ed), Marcel Dekker

West Virginia University, Department of Chemistry Website, Mass Spectroscopy. *Citing Internet resources* (www document).
http://www.chem.wuv.edu/chem210/#notes (accessed 12 February 2003)

White, P. A. (2002). The genotoxicity of priority polycyclic aromatic hydrocarbons in complex mixtures. *Mutation Research*, **515**, 85-98.

Wilson, G and Pahlavanpour, B (2000). Mineral insulating oil: a tool for maximising oil filled HV equipment lifetime. The National Grid Company Plc, *CIGRE*, 15-301.

Wong, P.K and Wang, J (2001) The accumulation of polycyclic aromatic hydrocarbons in lubricating oil over time- a comparison of supercritical fluid and liquid-liquid extraction methods. *Environmental Pollution*, **112**, 407-415

Yuspa, S.H and Bethesda, M.D (1986). Cutaneous chemical carcinogenesis. Jo*urnal of American Academic Dermatology*, **15, (5),** 1031-1044

Zeiger, E (2001). Mutagens that are not carcinogens: faulty theory or faulty tests? *Mutation Research,* **492**, 29-38

APPENDICES

APPENDIX A: ADDITIONAL MATERIALS

AMES TEST RECIPES Maron and Ames (1983)

Routine Checks to Ensure the Desired Mutations are Intact

Carried out on nutrient agar plates, once every 3 months or if the bacteria demonstrate unexpected characteristics.

Nutrient Agar Plates for Testing Genotypes:

Per litre:

- Nutrient broth 25g
- Agar 15g
- Distilled water 1000 ml

1. *Test For Crystal Violet Sensitivity* (rfa)

Per litre: (make up a few mL at a time)

- Crystal violet 0.1g
- Distilled water 100ml

Create well in the centre of a nutrient agar petri dish containing 0.1ml (approx. 10^8 bacteria) of nutrient broth culture to be tested in a thin overlay of agar (top agar). Add 4µl of the crystal violet solution to the well. After 12hrs incubation at 37^0C a clear zone of inhibition around the well (approx. 14mm diameter) indicates the presence of *rfa* mutation. *Alternatively 2mg of deoxycholate could be used instead of crystal violet.*

2. *Test for Presence of Ampicillin Resistant* R-Factor.

Per litre: (Make up a few mL at a time)

- Ampicillin trihydrate 0.8g
- Sodium hydroxide (0.02 N) 100ml

Create a well in the centre of the nutrient plate and add 4µl ampicillin. Incubate for 12-24 hrs at 37^0C R-factor strains will show no growth inhibition around the streak.

3. *Testing for Histidine Requirement:*

- Sterile 0.1M L-histidine 0.1ml
- 5mM biotin 0.1ml

Create a well in the centre of the minimal glucose plate and add 2µl of histidine and 2µl biotin.

Vogel-Bonner Solution:

Per Litre:

- 670mL Warm distilled water (45^0C)
- 10g Magnesium sulphate ($MgSO_4.7H_2O$)
- 100g Citric acid monohydrate
- 500g Potassium phosphate dibasic anhydrous (K_2HPO_4)
- 175g Sodium ammonium phosphate $NaHNH_4(PO_4.4H_2O)$

Add salts in order to warm water in 2L beaker or flask placed on magnetic stirrer hot plate. Allow each salt to dissolve completely before adding the next. Store at 4°C.

Sodium Phosphate Buffer for S-9 (PH 7.4)

Per 500mL:

- 60mL 0.2M sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$) (27.6 g mL^{-1})
- 440mL 0.2ml disodium hydrogen phosphate (Na_2HPO_4) (28.4 g mL^{-1})

Sterilize by autoclaving for 20 mins at 121°C.

0.5mM Histidine and Biotin Solution for Top Agar

Per 100 mL:

12.4 mg D-Biotin

9.6 mg L-Histidine.HCl

100 mL Distilled Water

IMMUNOASSAY KITS

The reagents supplied with the kit are as follows:

- PAH standards containing three concentrations of either phenanthrene (total kit; 2, 10 and 50 μg L^{-1}) or benzo[a]pyrene (carcinogenic kit; 0.1, 1 and 5 μg L^{-1}). These were used to obtain a calibration curve on which all unknown samples were quantified.
- PAH control containing 25 μg L^{-1} phenanthrene (total kit) or 2 μg L^{-1} benzo[a]pyrene (carcinogenic kit)
- A buffered saline diluent for the blank control and for diluting samples
- PAH analogue labelled with horeradish peroxidase (HRP) which was made up in buffered saline
- Magnetic particles covalently bonded to anti-PAH antibody in buffered saline
- Deionised water/detergent mixture to wash away excess reaction material
- A solution of hydrogen peroxide and 3,3′,5,5′-tetramethylbenzidine which acted as the enzyme substrate and produced an indicative colour change
- Sulphuric acid (0.5% v/v) to stop the reaction

APPENDIX B: ADDITIONAL METHODS

IP 346 METHOD

(Method BS2000 Part 346, 1996). All procedures were carried out in a fume cupboard with protective clothing and eyewear.

1. The DMSO was pre-equilibrate with cyclohexane by shaking 900 mL DMSO with 70 mL cyclohexane in a 250 mL separating funnel (with stopper) at room temperature for 30 seconds. The layers were left to separate for 10 minutes and the DMSO collected. The cyclohexane was discarded. The procedure was repeated until a sufficient amount of DMSO was obtained for the extraction. All references to DMSO in this protocol refer to pre-equilibrated DMSO.

2. A 3 mL volume of oil was added to a 250 mL separating funnel (with stopper) with 45 mL cyclohexane and 100 mL DMSO. The components were shaken vigorously (100 shakes) and left for 20 minutes to ensure complete layer separation. The DMSO layer was removed through a funnel (containing a plug of cotton wool) into a 1 L separating funnel.

3. To the 250 mL separating funnel, a fresh 100 mL portion of DMSO was added and step 2 was repeated.

4. To the 1 L separating funnel (containing the 2 x 100 mL DMSO fractions) 40 mL cyclohexane and 400 mL aqueous sodium chloride solution (4% w/v) was added. The stopper was replaced and the mixture was shaken vigorously (200 shakes) and left for 30 minutes to allow complete layer separation. The stopper was removed briefly after shaking 20 times to vent the pressure build up in the funnel, and replaced before shaking continued. The bottom layer (DMSO/NaCl) was removed to a second 1 L separating funnel. The cyclohexane was removed to a clean 250 mL separating funnel.

5. The DMSO/NaCl layer in the 1 L funnel was rinsed with 2 x 25 mL of cyclohexane, which was then added to the cyclohexane in the 250 mL funnel. A further 40 ml of cyclohexane was then added to the second 1 L funnel. The stopper was replaced and the mixture was shaken vigorously (200 shakes) and left for 30 minutes to allow complete layer separation. The lower layer was then discarded.

6. The remaining 40 mL cyclohexane was added to the cyclohexane fractions in the 250 mL funnel and washed with 2 x 25 mL sodium chloride solution (warmed to 70°C). The sodium chloride solution was discarded and the cyclohexane layer dried by passing it through a funnel containing filter paper and 5 g anhydrous sodium sulphate (Sigma-Aldrich). The cyclohexane was evaporated by rotary evaporator.

THE MINERAL INSULATING OIL FINGERPRINTING TECHNIQUE

The following was added to a 100 mL separating funnel:

5 g Oil sample

20 mL Cyclohexane

45 mL DMF

5 mL De-ionised water

The components were shaken vigorously (100 shakes) and left until the two layers were completely separate (~1 hour). The lower aqueous layer was retained.

To a clean 250 mL separating funnel the following was added:

Aqueous layer from previous separation

40 mL de-ionised water,

90 mL Cyclohexane

All of the above were shaken vigorously (100 shakes) and left to separate for ~30 minutes. The layers on some occasions were still cloudy and incomplete phase separation was evident, thus the lower aqueous layer was removed with great care (5-10 mL removed, then left to separate again for 5 minutes, before removing another 5-10 mL and repeated as required) until all of the lower layer was removed. The lower aqueous layer was then discarded.

A 15 mL volume of de-ionised water was then added to the residual material in the funnel to wash the organic layer. The aqueous phase was removed from the bottom of the funnel. This wash step was then repeated.

Filter paper was added to a glass funnel and a few grams of magnesium s ulphatewere placed onto the filter paper. The organic layer from the separating funnel was filtered through the filter paper/magnesium sulphate into a clean beaker to ensure all water had been removed. The solvent was finally evaporated with a rotary evaporator leaving a residual of aromatic material.

The next stage of the extraction was to pass the aromatic residue through a silica and C18 column for further clean up using cyclohexane.

APPENDIX C: THE AMES TEST RAW DATA

GRIMMER EXTRACTS WITH 10% S-9: REVERTANTS NUMBERS

Sample on Plate	Number of revertants Grimmer Extract 5g in triplicate			Number of revertants Grimmer Extract 0.5g			Number of revertants Grimmer Extract 0.25g			Number of revertants Grimmer Extract 0.05g			Number of revertants Grimmer Extract 0.025g		
White Oil without S-9	854	824	817	276	147	123	54	49	39	34	29	28	29	29	31
White Oil with S-9	758	759	842	275	258	186	38	35	34	25	24	26	32	31	34
Oil 4 without S-9	945	867	847	154	176	198	48	38	45	27	25	31	28	25	31
Oil 4 with S-9	857	869	845	125	124	100	28	32	35	30	29	26	29	36	34
Oil N10 without S-9	854	824	834	256	345	256	69	74	59	29	26	24	29	31	24
Oil N10 with S-9	945	1078	1088	356	367	356	94	68	75	38	35	34	34	31	31
Oil 8 without S-9	1104	1236	1344	278	193	167	68	75	74	35	36	38	28	29	31
Oil 8 with S-9	1010	1034	1231	134	152	125	32	29	31	32	28	36	35	28	31

IP 346 EXTRACTS WITH 10% S-9: REVERTANTS NUMBERS

Sample on Plate	Number of revertants IP 346 Extract 5g			Number of revertants IP 346 Extract 0.5g			Number of revertants IP 346 Extract 0.25g			Number of revertants IP 346 Extract 0.05g			Number of revertants IP 346 Extract 0.025g		
White Oil without S-9	34	35	36	34	38	26	34	35	30	31	29	28	34	30	31
White Oil with S-9	34	31	34	28	32	32	37	31	34	25	24	26	32	30	34
Oil 4 without S-9	34	32	35	29	35	29	34	32	29	29	28	34	31	28	31
Oil 4 with S-9	26	31	29	29	34	34	34	29	29	32	34	28	29	36	31
Oil N10 without S-9	35	32	34	34	29	34	35	32	34	29	26	24	29	30	30
Oil N10 with S-9	29	35	31	32	29	34	29	32	31	34	35	34	34	31	29
Oil 8 without S-9	34	35	34	30	31	30	30	35	34	30	36	38	29	34	35
Oil 8 with S-9	34	32	31	35	31	31	34	29	31	32	28	36	35	31	34

BLACKBURN WITH 80% S-9: REVERTANTS NUMBERS

Sample on Plate	Blackburn 5g Extract			Blackurn 0.5g Extract			Blackburn 0.05g Extract		
White Oil without S-9	1104	1310	1408	66	59	63	36	35	31
White Oil with S-9	1264	1244	1455	69	78	75	29	31	34
Oil 4 without S-9	1080	1568	1016	76	85	86	33	34	29
Oil 4 with S-9	1144	1088	1245	92	95	99	26	31	30
Oil N10 without S-9	784	1064	1204	66	75	78	38	35	36
Oil N10 with S-9	1856	1542	1695	66	75	79	35	35	33
Oil 8 without S-9	980	960	952	88	84	87	32	35	29
Oil 8 with S-9	1608	1552	1547	120	152	114	31	33	32
ALT 0 Oil without S-9	661	568	659	88	98	94	38	39	31
ALT 0 Oil with S-9	1524	1425	1253	123	118	128	32	35	39
ALT 1 Oil without S-9	960	944	945	85	88	79	32	31	34
ALT 1 Oil with S-9	1254	1348	1578	124	152	128	36	34	38
ALT 2 Oil without S-9	689	865	653	95	105	110	38	36	35
ALT 2 Oil with S-9	1224	1205	1015	103	129	115	34	33	38
ALT 3 Oil without S-9	702	652	625	99	109	105	38	35	31
ALT 3 Oil with S-9	1024	1058	1055	94	102	112	40	35	36

GRIMMER WITH 80% S-9: REVERTANTS NUMBERS

Sample on Plate	Grimmer 5g Extract			Grimmer 0.5g Extract		
White Oil without S-9	854	824	817	276	147	123
White Oil with S-9	845	855	835	245	259	164
Oil 4 without S-9	945	867	847	154	176	198
Oil 4 with S-9	986	984	934	178	202	176
Oil N10 without S-9	854	824	834	256	345	256
Oil N10 with S-9	1145	1178	1188	356	367	356
Oil 8 without S-9	1104	1236	1344	278	193	167
Oil 8 with S-9	1824	2150	3360	283	286	256
ALT 0 Oil without S-9	1289	1279	1352	165	167	130
ALT 0 Oil with S-9	1645	1520	1412	175	175	164
ALT 1 Oil without S-9	1451	1245	1542	167	156	129
ALT 1 Oil with S-9	1751	1542	1646	128	125	158
ALT 2 Oil without S-9	1254	1264	1228	167	111	132
ALT 2 Oil with S-9	1659	1547	1687	157	164	178
ALT 3 Oil without S-9	1225	1365	1244	68	171	131
ALT 3 Oil with S-9	1547	1547	1655	158	133	176

C18/SILICA/ISOLUTE PAH HC WITH 80% S-9

Sample on Plate	C18/silica/isolute 5 g Extract			C18/silica/isolute 2.5 g Extract			C18/silica/isolute 0.5 g Extract		
White Oil without S-9	39	32	41	47	35	36	29	34	32
White Oil with S-9	45	42	39	45	43	39	38	39	45
Oil 4 without S-9	32	34	31	31	32	31	35	35	39
Oil 4 with S-9	59	64	61	59	58	55	45	49	48
Oil N10 without S-9	39	34	40	30	31	35	32	34	31
Oil N10 with S-9	73	63	77	52	58	69	48	43	41
Oil 8 without S-9	32	35	42	29	30	32	31	30	31
Oil 8 with S-9	99	93	107	89	93	87	55	51	48
Blank Oil without S-9	35	35	32	31	32	31	35	32	34
Blank Oil with S-9	104	96	86	89	85	97	58	51	52
ALT 1 Oil without S-9	39	41	35	31	32	30	31	31	30
ALT 1 Oil with S-9	102	90	89	95	90	88	52	56	54
ALT 2 Oil without S-9	29	35	31	29	31	30	29	31	35
ALT 2 Oil with S-9	97	96	95	97	96	86	52	58	54
ALT 3 Oil without S-9	42	38	34	28	34	31	31	30	33
ALT 3 Oil with S-9	116	107	95	89	85	91	52	51	58

APPENDIX D: ION CHROMATOGRAM PEAK AREA FOR ESTIMATING TOTAL PAH CONTENT

TOTAL ION CHROMATOGRAM PEAK AREA

Oil	TIC Total Peak Integration			Mean	SD	%CV
1	1166817	1354264	1693607	1404896	267019.9	19.00638
2	9242183	11415561	12434566	11030770	1630606	14.78234
3	3382731	3648591	4542271	3857864	607437	15.74542
4	2516881	3147789	3772303	3145658	627713.7	19.95493
5	15269856	11535875	17108682	14638138	2839603	19.39866
6	6933755	7965452	9738563	8212590	1418642	17.27399
7	3717809	3209633	4501236	3809559	650671.3	17.07996
8	32945630	31578886	32689544	32404687	726536.3	2.242072
9	15900734	9960535	13564100	13141790	2992532	22.77112
10	14911454	12360952	17360952	14877786	2500170	16.80472
11	12656957	7424586	10615956	10232500	2637178	25.77256
12	17795273	17256864	17165350	17405829	340358.2	1.955426

SELECTIVE ION CHROMATOGRAM PEAK AREA

Oil	Total Peak integration of SIC			Mean	SD	%CV
1	639972	587904	678890	635588.7	45651.1	7.182492
2	194524	206954	184216	195231.3	11385.49	5.831795
3	435983	493903	284894.5	404926.8	107909.7	26.64918
4	179725	152816	202678.5	178406.5	24957.38	13.98906
5	2260732	1998219	2538585	2265845	270219.3	11.92576
6	1689805	1865972	1544903	1700227	160788.3	9.456872
7	1602865	1628666	1650098	1627210	23650.15	1.453418
8	4458392	4141791	4234987	4278390	162701.9	3.802877
9	1581507	1241518	1959714	1594246	359267.4	22.53525
10	4259110	5271253	5136808	4889057	549676.1	11.24299
11	1007150	1184153	1011728	1067677	100897.1	9.450156
12	1451025	1234518	1729030	1471524	247892.5	16.84597